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(57) Abstract

The present invention relates generally to genetic constructs which are useful in regulating the synthesis of malting enzymes in plants. More particularly, the present invention relates to nucleic acid molecules encoding gibberellin-regulated MYB polypeptides, for example the barley and rice *GAMyb* genetic sequences and transcriptionally-modulating parts and/or immunologically interactive parts thereof. The genetic sequences of the invention are introduced into plant cells, in particular monocotyledonous plant cells such as those derived from barley, wheat, maize, rye, rice or sorghum, where their expression in either the sense or antisense orientation modulates the expression of hydrolytic malting enzymes which are normally regulated by GAMYB polypeptides. The genetic sequences of the invention are therefore useful in the production of plants with altered malting properties.

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"PLANT REGULATORY PROTEINS III"

The present invention relates generally to genetic constructs useful in regulating the synthesis of malting enzymes in plants, such as the α-amylase enzyme. More particularly, the present invention relates to nucleic acid molecules encoding gibberellin-regulated MYB polypeptides, for example the barley and rice aleurone gibberellin-regulated MYB polypeptides, and transcriptionally modulating parts and/or immunologically interactive parts thereof.

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description. Sequence identity numbers (SEQ ID NOs.) for the nucleotide and amino acid sequences referred to in the specification are defined after the bibliography.

Throughout the specification and the claims that follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising" will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Malting and brewing are multi-million dollar industries. The annual added-value on barley production alone was approximately US$3 billion, in the USA between 1983 and 1992. During the same period, the US brewing industry generated US$167 billion in total business activity. The economic benefits to be derived from increased productivity of the brewing industry worldwide, are enormous. A major contributing factor to this increased productivity is the improvement of the input raw material, the barley crop, the quality of which is largely determined by its genetic profile.

The primary goal of barley improvement for use in the brewing industry is to expand the germplasm available to breeders, thereby making available elite cultivars which are higher yielding, disease resistant, and/or possess improved malting characteristics. Traditional plant breeding methods have made considerable progress toward these goals, however such processes are labour-intensive, imprecise and protracted, requiring several generations of genetic crosses to produce a substantially-improved genetic stock. The present invention is a significant advance in the improvement of malting characteristics of plant materials utilised in the brewing industry, in particular barley, wherein said invention provides a "master switch", controlling the expression of several malting genes, for example α-amylase and β-glucanase, amongst others.

During the malting process, the barley grain is water-steeped for 1-2 days at 10-20°C to remove CO_2, replace oxygen and dissipate heat. The steeping process induces germination of the seed, characterised by cell elongation and increased respiration in the embryo, stimulation of embryo secretions, protein biosynthesis and enzyme activation, and the initiation of endosperm hydration. Kernel moisture content increases from 10-15% (w/w) to 40-45% (w/w) as a result of steeping (botanically-defined germination initiates at approximately 30% moisture content). Following steeping, the grains are germinated in a controlled growth environment for 3-6 days, producing a "green malt". Losses may be incurred at this stage, from incomplete or variable germination of the seed and consequently, there are many benefits to be derived from producing a crop in which seed dormancy could be broken uniformly and completely during steeping, providing rapid and uniform germination. Furthermore, dormancy of barley seed should also be adequate to prevent pre-harvest sprouting and consequential crop losses. Accordingly, the present invention provides a means of regulating the expression of the α-amylase gene in aleurone cells of the seed. The present invention may be used to control the germination of a crop seed.

During malting, the major constituent of the cell wall of the starchy endosperm of barley, β-glucan, is broken down by the enzyme (1-3, 1-4)-β-glucanase, referred to hereinafter as β-glucanase, which is secreted from the aleurone cells (Fincher, 1989). The efficient

degradation of β-glucan polymers is important to the brewing process, since the presence of high molecular weight β-glucans increases the viscosity of the "mash", thereby slowing later filtration steps. Incomplete hydrolysis of β-glucan molecules may even produce a cloudy precipitate in the finished product. The degree of hydrolysis of β-glucan is a function of the level of β-glucanase enzyme produced by the aleurone and the proportion of enzyme activity remaining following higher temperature incubations of kilning and mashing (see below). There is a clear need in the malting industry for the production of barley lines with increased β-glucanase activity, to facilitate the malting process.

The green malt is dried in kilns to reduce kernel moisture to 3-5%. Malt components are subsequently converted into a fermentable substrate which includes sugars, amino acids, nucleic acids, vitamins and minerals. The malt is placed in warm water and taken through a series of controlled temperature rises and holds from 40°C to 75°C, in order to gelatinise and solubilise seed starch reserves and to solubilise carbohydrate-degrading enzymes. The production of fermentable sugars for example maltose and glucose, requires the hydrolytic enzymes α-amylase, β-amylase, α-glucosidase and limit dextrinase. Of these enzymes, α-amylase, α-glucosidase and limit dextrinase are known to be secreted from the aleurone (Fincher, 1989).

Fermentable sugars are produced by α-amylase enzyme activity at 70-75°C. The survival of these hydrolytic enzymes, in particular α-amylase, during and after kilning, is critical to the brewing process and to the flavour and colour of the malt and the alcohol content of the finished product. The proportion of these enzymes remaining after kilning is directly proportional to the amount of enzyme in the germinating seed after dormancy is broken. Using technology available until the present invention, the efficiency of this process was improved by the addition of a microbial amyloglucosidase supplement to the mash, in particular in the production of some low calorie beers.

Although the development of a barley crop with improved malting characteristics, in particular possessing increased aleurone α-amylase, α-glucosidase, limit dextrinase, β-

glucanase, endoxylanase and protease activities, is highly desirable, traditional breeding technologies have not addressed the problem, in part because reliable methods for screening large numbers of plants carrying these trials have not been developed.

The level of aleurone secretory enzymes, in particular α-amylase, β-glucanase, etc., may be increased by the application of the plant hormone, gibberellic acid (GA), in particular GA_3 (Paleg, 1960; Varner, 1964; Yomo, 1960). Following addition of GA, there is a rapid rise in α-amylase gene expression in isolated barley aleurone layers and this effect is inhibited by ascisic acid (Jacobsen *et al.*, 1995).

Although there are now considerable data about the site of GA perception in aleurone cells, the GA receptor has not yet been identified. Evidence from experiments using GA_4 covalently bound to Sepharose beads and anti-idiotype antibodies suggests that GA is perceived on the plasma membrane in oat aleurone protoplasts (Hooley *et al.*, 1991; Hooley *et al.*, 1992). This is supported by recent work which shows micro injection of GA_3 into isolated barley aleurone protoplasts failed to induce α-amylase synthesis and secretion (Gilroy and Jones, 1994). However, when GA_3 was applied external to the medium, the protoplasts responded by increasing α-amylase gene expression, indicating that the site of perception is on the external face of the plasma membrane. Little is known of the molecular events downstream of the GA receptor which transmit the GA signal through the cytoplasm and ultimately trigger expression of genes encoding α-amylase and other hydrolytic enzymes (Bush and Jones, 1990; Gilroy and Jones 1992).

Functional analysis of barley high-pI α-amylase promoters have identified a gibberellin response complex (GARC) consisting of the pyrimidine, TAACAAA and TATCCAC boxes, which mediate the GA response (Skriver *et al.*, 1991; Gubler and Jacobsen, 1992; Gubler *et al.*, 1995). There is also evidence that the action of abscisic acid is mediated *via* the same complex. Analyses of a barley low-pI amylase promoter, have shown that GA probably also acts through similar *cis*-acting elements, but additional *cis*-acting elements upstream of the pyrimidine box are also important (Lanahan *et al.*, 1992). DNA sequences that bind nuclear proteins *in vitro* have been identified in cereal α-

amylase promoters using *DNase* 1 foot printing and gel mobility shift assays (Ou-Lee *et al.*, 1988; Rushton *et al.*, 1992; Sutliff *et al.*, 1993; Goldman *et al.*, 1994). Two recent studies have shown that GARC sequences in wheat and barley α-amylase promoters can act as binding sites for nuclear transcription factors which regulate gene expression. Sutliff *et al.* (1993) characterized nuclear factors from barley aleurone layers which bound *in vitro*, to sequences from a barley low-pI α-amylase gene. A GA-dependent binding factor was shown to bind specifically to sequences which coincide with the TAACAGA and TATCCAT boxes and proximal sequences. It is not yet clear whether this binding factor contains a single nuclear protein which binds to both elements or whether it consists of two or more proteins with different binding specificities. Rushton *et al.* (1992) demonstrated that nuclear factors from GA-treated oat protoplasts bound specifically to the box 2 and the pyrimidine and TAACAGA elements in a low-pI wheat α-amylase promoter. The function of these proteins in regulating α-amylase gene expression has not been determined. Furthermore, the demonstration of a DNA-protein interaction does not provide significant direction to enable a person normally skilled in the art to isolate the DNA-binding protein or a gene encoding said protein, or to determine the role of said protein in regulating the expression of genes encoding malting enzymes, for example α-amylase and β-glucanase, amongst others.

One aspect of the present invention provides an isolated nucleic sequence which encodes, or is complementary to a sequence which encodes, a seed-specific gibberellin-regulated MYB polypeptide, seed-expressed gibberellin-regulated MYB polypeptide or other gibberellin-regulated MYB polypeptide.

Preferably, said MYB regulates the expression of gibberellin-regulated genes encoding hydrolytic enzymes involved in the malting process, for example high pI α-amylase, low pI α-amylase, EII-(1-3,1-4)-β-glucanase, Cathepsin β-like proteases, α-glucosidase, xylanase, arabinofuranosidases, amongst others.

Preferably, the isolated nucleic acid molecule of the invention is cDNA, genomic DNA or mRNA. In a particularly preferred embodiment, the nucleic acid molecule is a cDNA

molecule.

In a preferred embodiment of the invention, the isolated nucleic acid molecule of the invention is derived from a monocotyledonous plant species selected from the list comprising rice, barley, wheat, maize, rye and sorghum, amongst others. In a particularly preferred embodiment, the isolated nucleic acid molecule is derived from barley or rice.

The present invention extends to the isolated nucleic acid molecule when integrated into the genome of a cell as an addition to the endogenous cellular complement of gibberellin-regulated *Myb* genes. The said integrated nucleic acid molecule may, or may not, contain promoter sequences which confer gibberellin-regulated expression of the *Myb* genetic sequence contained therein.

Hereinafter the term "gibberellin-regulated MYB", or "GAMYB", or similar term shall refer to a polypeptide belonging to the class of MYB transcription factors, the synthesis and/or activity of which is normally regulated by gibberellins in the cells of plants, in particular in seeds or germinating seedlings, and which control the expression of genes involved in:

(i) plant developmental processes selected from the list comprising stem elongation, flowering, leaf development, fruit set and growth, sex determination, germination, amonst others; or

(ii) malting characteristics selected from the list comprising seed dormancy, germination, post-kilning levels of hydrolytic enzymes, mash filtration properties, precipitate formation and alcohol content, amongst others, wherein said hydrolytic enzymes are selected from the list comprising GA-regulated hydrolases, in particular the high pI α-amylase, low pI α-amylase, EII-(1-3,1-4)-β-glucanase and Cathepsin β-like proteolytic enzymes, amongst others.

A GAMYB as defined herein may further be regulated by other plant hormones, for

example abscisic acid, amongst others.

Hereinafter the term "gibberellin-regulated *Myb* gene", "*GAMyb*" or similar term shall be used to define a gene which upon expression, encodes a polypeptide comprising an amino acid sequence having the activity of a gibberellin-regulated MYB polypeptide.

Reference herein to "genes" is to be taken in its broadest context and includes:

(I) a classical genomic gene consisting of transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (i.e. introns, 5'- and 3'- untranslated sequences); or

(ii) mRNA or cDNA corresponding to the coding regions (i.e. exons) and 5'- and 3'- untranslated sequences of the gene.

The term "gene" is also used to describe synthetic or fusion molecules encoding all or part of a functional product. Preferred gibberellin-regulated *Myb* genes may be derived from a naturally-occurring gibberellin-regulated *Myb* gene by standard recombinant techniques. Generally, a gibberellin-regulated *Myb* gene may be subjected to mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or additions. Nucleotide insertional derivatives of the gibberellin-regulated *Myb* gene of the present invention include 5' and 3' terminal fusions as well as intra-sequence insertions of single or multiple nucleotides. Insertional nucleotide sequence variants are those in which one or more nucleotides are introduced into a predetermined site in the nucleotide sequence although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more nucleotides from the sequence. Substitutional nucleotide variants are those in which at least one nucleotide in the sequence has been removed and a different nucleotide inserted in its place. Such a substitution may be "silent" in that the substitution does not change the amino acid defined by the codon. Alternatively, substituents are designed to alter one amino acid for another similar acting amino acid, or amino acid of like charge, polarity, or hydrophobicity.

Another aspect of the present invention is directed to an isolated nucleic molecule which comprises a sequence of nucleotides corresponding, or complementary to the sequence set forth in SEQ ID NO:1 or SEQ ID NO:3 or a homologue, analogue or derivative thereof, or having at least about 40%, more preferably at least about 55%, still more preferably at least about 65%, yet still more preferably at least about 75-80% and even still more preferably at least about 85-95% nucleotide similarity to all, or a part thereof, of SEQ ID NO:1 or SEQ ID NO:3.

According to this aspect, said nucleic acid molecule encodes, or is complementary to a nucleotide sequence encoding, a plant gibberellin-regulated MYB polypeptide.

In a preferred embodiment, the nucleic acid molecule is derived from a monocotyledonous plant species selected from the list comprising rice, barley, wheat, maize, rye and sorghum. In a particularly preferred embodiment, the isolated nucleic acid molecule is derived from barley or rice.

For the purposes of nomenclature, the nucleotide sequence shown in SEQ ID NO: 1 relates to the barley *GAMyb* cDNA sequence (*HvGAMyb*), which is expressed in barley aleurone cells in response to exogenous application of gibberellin. Preferably, the polypeptide encoded therein regulates expression of a number of genes involved in the malting process, including α-amylases, in particular high pI α-amylases and low pI α-amylases, β-glucanases, in particular EII-(1-3,1-4)-β-glucanase, proteases, in particular Cathepsin β-like proteases, α-glucosidases, xylanases, and arabinofuranosidases, amongst others. More preferably, the polypeptide encoded therein, regulates, at least the expression of the barley α-amylase gene, in aleurone cells.

The nucleotide sequence set forth in SEQ ID NO:3 relates to the rice homologue of the barley *GAMyb* cDNA sequence, designated hereinafter as "rice *GAMyb*" or "*OsGAMyb*". Details of the isolation and characterisation of the rice *GAMyb* cDNA clone are provided by the Examples incorporated herein.

For the present purpose, "homologues" of a nucleotide sequence shall be taken to refer to an isolated nucleic acid molecule which is functionally the same as the nucleic acid molecule of the present invention or its complementary nucleotide sequence, notwithstanding the occurrence within said sequence, of one or more nucleotide substitutions, insertions, deletions, or rearrangements.

"Analogues" of a nucleotide sequence set forth herein shall be taken to refer to an isolated nucleic acid molecule which is substantially the same as a nucleic acid molecule of the present invention or its complementary nucleotide sequence, notwithstanding the occurrence of any non-nucleotide constituents not normally present in said isolated nucleic acid molecule, for example carbohydrates, radiochemicals including radionucleotides, reporter molecules such as, but not limited to DIG, alkaline phosphatase or horseradish peroxidase, amongst others.

"Derivatives" of a nucleotide sequence set forth herein shall be taken to refer to any isolated nucleic acid molecule which contains significant sequence similarity to said sequence or a part thereof. Generally, the nucleotide sequence of the present invention may be subjected to mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or insertions. Nucleotide insertional derivatives of the nucleotide sequence of the present invention include 5′ and 3′ terminal fusions as well as intra-sequence insertions of single or multiple nucleotides or nucleotide analogues. Insertional nucleotide sequence variants are those in which one or more nucleotides or nucleotide analogues are introduced into a predetermined site in the nucleotide sequence of said sequence, although random insertion is also possible with suitable screening of the resulting product being performed. Deletional variants are characterised by the removal of one or more nucleotides from the nucleotide sequence. Substitutional nucleotide variants are those in which at least one nucleotide in the sequence has been removed and a different nucleotide or nucleotide analogue inserted in its place.

A further aspect of the present invention provides an isolated nucleic molecule which is capable of hybridising under at least low stringency conditions to the nucleic acid

molecule set forth in SEQ ID NO:1 or SEQ ID NO:3, or to a complementary strand or a homologue, analogue or derivative thereof.

Preferably, said nucleic acid molecule encodes, or is complementary to a nucleic acid molecule which encodes a plant gibberellin-regulated MYB polypeptide.

More preferably, the nucleic acid molecule encodes, or is complementary to a nucleic acid molecule which encodes, a gibberellin-regulated MYB polypeptide and which is capable of hybridising under at least low stringency conditions to all, or a part thereof, nucleotide residues 1 to 400 or residues 710 to 2220, of the nucleic acid molecule set forth in SEQ ID NO: 1, or to a complementary strand or a homologue, analogue or derivative thereof.

In a particularly preferred embodiment, said nucleic acid molecule does not encode solely, or is not complementary to a nucleic acid molecule which encodes solely, the conserved R2 and R3 domains of a MYB polypeptide of plant origin or a part thereof, or does not hybridise exclusively under low stringency conditions to nucleotide residues 401 to 709, of the nucleic acid molecule set forth in SEQ ID NO: 1 encoding same, or to a complementary strand thereof.

In an alternative preferred embodiment, the nucleic acid molecule encodes, or is complementary to a nucleic acid molecule which encodes, a gibberellin-regulated MYB polypeptide and which is capable of hybridising under at least low stringency conditions to all, or a part thereof, nucleotide residues 1 to 671 or residues 816 to 2352 of the nucleic acid molecule set forth in SEQ ID NO:3, or to a complementary strand or a homologue, analogue or derivative thereof, which do not encode, or are not complementary to a nucleic acid molecule which encodes the conserved R2 and R3 domains of a MYB polypeptide.

Accordingly, the isolated nucleic acid molecule according to this preferred embodiment does not hybridise exclusively under low stringency conditions to nucleotide residues 672 to 815 of the nucleic acid molecule set forth in SEQ ID NO: 3 or to a complementary

strand thereof.

For the purposes of defining the level of stringency, a low stringency is defined herein as being a hybridisation and/or a wash carried out in 6xSSC buffer, 0.1% (w/v) SDS at a temperature in the range 28°C-55°C. Generally, the stringency is increased by reducing the concentration of SSC buffer, and/or increasing the concentration of SDS and/or increasing the temperature of the hybridisation and/or wash. Conditions for hybridisations and washes are well understood by one normally skilled in the art. For the purposes of clarification, of the parameters affecting hybridisation between nucleic acid molecules, reference is found in pages 2.10.8 to 2.10.16. of Ausubel *et al.* (1987), which is herein incorporated by reference.

The present invention is particularly directed to a gibberellin-regulated *Myb* gene, such as the barley *GAMyb* or rice *GAMyb*. The subject invention clearly contemplates other sources of gibberellin-regulated *Myb* genes, or gibberellin-regulated *Myb*-like genes, such as but not limited to, aleurone or embryo tissues and cultured cells of plant origin, derived from, but not limited to, other plant species including, wheat, maize, rye, or sorghum. Preferably, said *Myb* gene, or *Myb*-like gene, is involved in the transcriptional modulation of genes involved in biological processes selected from the list comprising, but not limited to, stem elongation, flowering, leaf development, fruit set and growth, sex determination, germination or malting characteristics as hereinbefore defined.

The present invention clearly contemplates a genomic clone equivalent of the nucleotide sequence set forth in SEQ ID NO:1 or SEQ ID NO:3 and extends to a promoter or functional derivative, part fragment, homologue or analogue thereof from a genomic clone equivalent of the nucleotide sequence defined by SEQ ID NO:1 or SEQ ID NO:3.

The genetic sequences which encode, or are complementary to genetic sequences which encode, a gibberellin-regulated MYB polypeptide or gibberellin-regulated MYB-like polypeptide, may correspond to the naturally occurring sequence or may differ by one or more nucleotide substitutions, deletions and/or additions. Accordingly, the present

invention extends to gibberellin-regulated *Myb* and *Myb*-like genes and any functional genes, mutants, derivatives, parts, fragments, homologues or analogues thereof or non-functional molecules but which are at least useful as, for example, genetic probes, or primer sequences in the enzymatic or chemical synthesis of said gene, or in the generation of immunologically interactive recombinant molecules.

In a particularly preferred embodiment, the gibberellin-regulated *Myb* genetic sequences disclosed herein are employed to identify and isolate similar genes from other barley or rice cells, tissues, or organ types, or from the cells, tissues, or organs of other plant species.

According to this embodiment, there is contemplated a method for identifying a related gibberellin-regulated *Myb* genetic sequence, or gibberellin-regulated *Myb*-like genetic sequence, said method comprising contacting genomic DNA, or mRNA, or cDNA with a hybridisation effective amount of a gibberellin-regulated *Myb* genetic sequence, or a functional part, homologue, analogue or derivative thereof, and then detecting said hybridisation.

The related genetic sequence may be in a recombinant form, in a virus particle, bacteriophage particle, yeast cell, animal cell, or a plant cell. Preferably, the related genetic sequence originates from a plant species other than the species from which the gibberellin-regulated *Myb* genetic sequence was derived. More preferably, the related genetic sequence originates from a plant used in the malting process, for example the monocotyledonous plants wheat, rye, maize, rice or sorghum, amongst others. In a particularly preferred embodiment the related genetic sequences are derived from rice.

Preferably, the gibberellin-regulated *Myb* genetic sequence (i.e latter genetic sequence) is from a monocotyledonous plant species. In a most preferred embodiment, the latter genetic sequence is as set forth in SEQ ID NO:1 or SEQ ID NO:3.

Preferably, the latter genetic sequence is labelled with a reporter molecule capable of

giving an identifiable signal (e.g. a radioisotope such as ^{32}P or ^{35}S or a biotinylated molecule).

An alternative method contemplated in the present invention involves hybridising two nucleic acid "primer molecules" of at least 15 nucleotides in length to a nucleic acid "template molecule", said template molecule herein defined as a related gibberellin-regulated *Myb* genetic sequence, or a functional part thereof, or its complementary sequence. Specific nucleic acid molecule copies of the template molecule are amplified enzymatically in a polymerase chain reaction, a technique that is well known to one skilled in the art.

Preferably, the nucleic acid primer molecules are contained in an aqueous mixture of other nucleic acid primer molecules. More preferably, the nucleic acid primer molecule is in a substantially pure form. In a preferred embodiment, each nucleic acid primer molecule is any nucleotide sequence of at least 15 nucleotides in length derived from, or complementary to the nucleotide sequence set forth in SEQ ID NO:1 or SEQ ID NO:3, or a homologue, analogue or derivative thereof.

In a particularly preferred embodiment, at least one primer molecule is substantially the same as, or complementary to, nucleotide sequences comprising at least 15 nucleotides in length, of the sequences set forth in residues 1 to 400, or 710 to 2220 of SEQ ID NO: 1 or alternatively, nucleotide residues 1 to 671 or 816 to 2352 of SEQ ID NO:3, which do not encode highly conserved R2 or R3 amino acid sequence motifs found in MYB polypeptides. According to this embodiment, the nucleic acid primer molecule consists of a combination of any of the nucleotides adenine, cytidine, guanine, thymidine, or inosine, or functional analogues or derivatives thereof, capable of being incorporated into a polynucleotide molecule.

The nucleic acid template molecule may be in a recombinant form, in a virus particle, bacteriophage particle, yeast cell, animal cell, or a plant cell. Preferably, the related

genetic sequence originates from a mammalian cell, tissue, or organ. More preferably, the related genetic sequence originates from a plant cell, tissue or organ.

Yet another aspect of the present invention provides for the expression of the subject genetic sequence in a suitable host (e.g. a prokaryote or eukaryote) to produce full length or non-full length recombinant gibberellin-regulated MYB gene products. Preferably, the gibberellin-regulated MYB gene product has a sequence that is identical to, or contained within the amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:4.

For the purposes of nomenclature, the amino acid sequences set forth in SEQ ID Nos:2 and 4 relate to the barley and rice GAMYB polypeptides, respectively.

In an alternative embodiment, the present invention provides an isolated polypeptide which comprises an amino acid sequence having the transcriptional activation function of a gibberellin-regulated MYB, or a functional homologue, mutant, derivative, part, fragment, or analogue of said polypeptide.

Preferably, the polypeptide is the polypeptide product of the barley or rice *GAMyb* gene sequence.

In the present context, "homologues" of an amino acid sequence refer to those polypeptides, enzymes or proteins which have a similar catalytic activity to the amino acid sequences set forth in SEQ ID NO:2 or SEQ ID NO:4, notwithstanding any amino acid substitutions, additions or deletions thereto. A homologue may be isolated or derived from the same or another plant species as the species from which the polypeptides of the invention are derived.

Furthermore, the amino acids of a homologous polypeptide may be replaced by other amino acids having similar properties, for example hydrophobicity, hydrophilicity, hydrophobic moment or antigenicity, and so on.

"Analogues" encompass polypeptides of the invention notwithstanding the occurrence of any non-naturally occurring amino acid analogues therein.

The term "derivative" in relation to a GAMYB polypeptide shall be taken to refer hereinafter to mutants, parts or fragments of the complete barley or rice GAMYB polypeptides defined herein. Derivatives include modified peptides in which ligands are attached to one or more of the amino acid residues contained therein, such as carbohydrates, enzymes, proteins, polypeptides or reporter molecules such as radionuclides or fluorescent compounds. Glycosylated, fluorescent, acylated or alkylated forms of the subject peptides are particularly contemplated by the present invention. Additionally, derivatives of SEQ ID NO:2 or SEQ ID NO:4 which comprise fragments parts of the subject amino acid sequences are within the scope of the invention, as are homopolymers or heteropolymers comprising two or more copies of the subject polypeptides. Procedures for derivatizing peptides are well-known in the art.

Substitutions encompass amino acid alterations in which an amino acid is replaced with a different naturally-occurring or a non-conventional amino acid residue. Such substitutions may be classified as "conservative", in which an amino acid residue contained in a cellulose gene product is replaced with another naturally-occurring amino acid of similar character, for example Gly↔Ala, Val↔Ile↔Leu, Asp↔Glu, Lys↔Arg, Asn↔Gln or Phe↔Trp↔Tyr.

Substitutions encompassed by the present invention may also be "non-conservative", in which an amino acid residue which is present in a cellulose gene product described herein is substituted with an amino acid with different properties, such as a naturally-occurring amino acid from a different group (eg. substituted a charged or hydrophobic amino acid with alanine), or alternatively, in which a naturally-occurring amino acid is substituted with a non-conventional amino acid. Non-conventional amino acids encompassed by the invention include, but are not limited to those listed in Table 2.

Amino acid substitutions are typically of single residues, but may be of multiple residues,

either clustered or dispersed.

Amino acid deletions will usually be of the order of about 1-10 amino acid residues, while insertions may be of any length. Deletions and insertions may be made to the N-terminus, the C-terminus or be internal deletions or insertions. Generally, insertions within the amino acid sequence will be smaller than amino-or carboxyl-terminal fusions and of the order of 1-4 amino acid residues.

The present invention extends to a gibberellin-regulated *Myb* gene characterised by said gene encoding a gibberellin-regulated MYB polypeptide having at least two similar amino acid repeat regions comprising 2 to 3 conserved tryptophan residues spaced 18 to 19 residues apart in each repeat region, hereinafter defined as structural features designated "R2" and "R3". While not wishing to be bound by any theory or mode of action, the conserved tryptophan residues may play a critical role in stabilising the DNA-binding domain of MYB transcription factors (Ogata *et al.*, 1992).

Although the R2 and R3 motifs are conserved in MYB-related proteins, they provide no indication of the role of a particular MYB polypeptide in a cell and, since MYB-related proteins are numerous, it is not a straightforward procedure to use these high-conserved motifs to isolate a specific MYB-related polypeptide, or cDNA clone encoding same, which is involved in the transcriptional activation of a known gene, for example α-amylase.

The present invention extends further to a gibberellin-regulated *Myb* gene encoding a polypeptide which contains conserved R2 and R3 domains having at least approximately 85% identity to the R2 and R3 domains of *HvGAMyb* or *OsGAMyb*, with very low sequence identity outside these regions. Such GAMYB polypeptides bind to the same GA-response element, TAACAAA box or TAACAAA-like box to which barley and rice GAMYB polypeptides bind.

Preferably, the R2 and R3 structural features are adjacent and located at, or near, the N-

terminal end of a GAMYB polypeptide and have at least 85% similarity to amino acid residues 42 to 145 of the amino acid sequence set forth in SEQ ID NO: 2 or to amino acid residues 93 to 143 of SEQ ID NO:4. More preferably, the R2 and R3 regions are substantially the same as amino acid residues 42 to 145, of the amino acid sequence set forth in SEQ ID NO: 2 or amino acid residues 93 to 143 of SEQ ID NO:4.

In a related embodiment, the present invention provides an isolated polypeptide which:

(I) contains structural features R2 and R3 of a gibberellin-regulated MYB polypeptide; and

(ii) has at least 40% amino acid sequence similarity to the sequence set forth in SEQ ID NO:2 or SEQ ID NO:4 other than the R2 or R3 domains, or a homologue, analogue, derivative or part thereof.

Preferably, the percentage amino acid similarity is at least 60%, more preferably at least 80% and even more preferably at least 90%, including 91%, 93% or 95%.

In a particularly preferred embodiment, the present invention provides an isolated polypeptide which:

(I) contains structural features R2 and R3 of a gibberellin-regulated MYB polypeptide, wherein said features comprise an amino acid sequence which is at least 85% identical to amino acid residues 42 to 145 of SEQ ID NO: 2 or residues 93 to 143 of SEQ ID NO:4 ; and

(ii) has at least 75% amino acid sequence similarity to the sequence set forth in SEQ ID NO:2 or SEQ ID NO:4 other than said features, or a homologue, analogue, derivative or part thereof.

In a related embodiment, the present invention provides a "sequencably pure" form of the amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:4. "Sequencably pure" is hereinbefore described as substantially homogeneous to facilitate amino acid determination. In a further related embodiment, the present invention provides a

"substantially homogeneous" form of the amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:4, wherein the term "substantially homogeneous" is hereinbefore defined as being in a form suitable for interaction with an immunologically interactive molecule. Preferably, the polypeptide is at least 20% homogeneous, more preferably at least 50% homogeneous, still more preferably at least 75% homogeneous and yet still more preferably at least about 95-100% homogenous, in terms of activity per microgram of total protein in the protein preparation.

The present invention also extends to a synthetic peptide comprising any part of the amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:4, or having at least 40% similarity to all or a part thereof, wherein the amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:4 is a gibberellin-regulated MYB polypeptide.

In a particularly preferred embodiment, the invention provides a synthetic peptide comprising amino acid residues 1 to 14 of SEQ ID NO:2 or amino acid residues 481 to 494 of SEQ ID NO:2 or a homologue, analogue or derivative thereof.

In a related embodiment, the invention provides a synthetic peptide comprising the amino acid sequence:

(I) MYRVKSESDCEMMHC; or

(ii) CGAGDTSSHPENLRP,

or a homologue, analogue or derivative thereof.

The recombinant gibberellin-regulated MYB gene product, or part fragment, functional derivative, synthetic peptide, or 3-dimensional structure thereof, is used to produce immunologically interactive molecules, such as antibodies, or functional derivatives thereof, for example Fabs, SCABS (single-chain antibodies), or antibodies conjugated to an enzyme, radioactive or fluorescent tag, the only requirement being that the recombinant products are immunologically interactive with antibodies to all, or part, or a gibberellin-regulated MYB gene product.

According to this aspect, the present invention provides an antibody that binds to a polypeptide comprising an amino acid sequence which:

(I) has the transcriptional activation function of a gibberellin-regulated MYB, or a functional mutant, derivative, part, fragment, or analogue of said polypeptide; and

(ii) is substantially the same as the amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO:4, or has at least 40% similarity to all or a part thereof.

In a particularly preferred embodiment, antibodies are raised against a synthetic peptide as hereinbefore defined and are capable of binding to the amino acid sequence:

(I) MYRVKSESDCEMMHC; or

(ii) CGAGDTSSHPENLRP,

or a homologue, analogue or derivative thereof.

Antibodies to a recombinant gibberellin-regulated MYB polypeptide are particularly useful in screening to isolate related gibberellin-regulated *Myb* genetic sequences and gibberellin-regulated *Myb*-like genetic sequences. The only requirements for successful detection of a related gibberellin-regulated *Myb* genetic sequence, or gibberellin-regulated *Myb*-like genetic sequence are that the said genetic sequence is expressed to produce a polypeptide, wherein said polypeptide contains at least one epitope recognised by an antibody molecule that binds to a gibberellin-regulated MYB polypeptide.

Preferably, for the purpose of obtaining expression to facilitate detection, the related gibberellin-regulated *Myb* genetic sequence, or gibberellin-regulated *Myb*-like genetic sequence is placed operably behind a promoter sequence, for example the bacterial *lac* promoter. According to this preferred embodiment, the antibodies that bind to a gibberellin-regulated MYB polypeptide are employed to detect the presence of a plasmid or bacteriophage which is capable of expressing said related gibberellin-regulated *Myb* genetic sequence, or gibberellin-regulated-*Myb*-like genetic sequence and are therefore useful in purifying the same.

Such antibodies may be monoclonal or polyclonal and may be selected from naturally occurring antibodies to an epitope, or peptide fragment, or synthetic peptide of a gibberellin-regulated MYB gene product or may be specifically raised against a recombinant gibberellin-regulated MYB gene product. Both polyclonal and monoclonal antibodies are obtainable by immunisation with an appropriate gene product, or epitope, or peptide fragment of a gene product. Alternatively, fragments of antibodies may be used, such as Fab fragments. Furthermore, the present invention extends to recombinant and synthetic antibodies and to antibody hybrids. A "synthetic antibody" is considered herein to include fragments and hybrids of antibodies.

The nucleic acid molecule of the present invention is also useful for developing genetic constructs which express a gibberellin-regulated *Myb* genetic sequence, thereby providing for the increased expression of genes encoding hydrolytic malting enzymes, such as, but not limited to, α-amylase, β-glucanases such as EII-(1-3,1-4)-β-glucanase, proteases such as Cathepsin β-like protease, α-glucosidase, xylanase, arabinofuranosidase, amongst others.

According to this embodiment, the coding region of a gibberellin-regulated *Myb* gene is placed operably behind a promoter, in the sense orientation, such that a MYB polypeptide is capable of being expressed under the control of said promoter sequence.

In a particularly preferred embodiment, the gibberellin-regulated *Myb* genetic sequence is the barley *GAMyb* genetic sequence set forth in SEQ ID NO: 1 or the rice *GAMyb* genetic sequence set forth in SEQ ID NO: 3.

The nucleic acid molecule of the present invention is also useful for developing genetic constructs employing antisense or ribozyme molecules, or in co-suppression of the gibberellin-regulated *Myb* gene, in particular the barley or rice *GAMyb* genes. By targeting the endogenous gibberellin-regulated *Myb* gene, expression is diminished, reduced or otherwise lowered to a level that results in reduced expression of malting enzymes, in particular α-amylase.

Preferably, the reduced expression of the *GAMyb* genetic sequence also reduces expression of genes encoding other malting enzymes, for example α-amylases such as high pI and low pI α-amylases, β-glucanase such as EII-(1-3,1-4)-β-glucanase, proteases such as Cathepsin β-like protease, α-glucosidase, xylanase, arabinofuranosidase, amongst others.

Co-suppression is the reduction in expression of an endogenous gene that occurs when one or more copies of said gene, or one or more copies of a substantially similar gene are introduced into the cell. The present invention also extends to the use of co-suppression to inhibit the expression of a gene which encodes a gibberellin-regulated MYB polypeptide.

Preferably, the gene which is targeted by a co-suppression molecule, is the barley *GAMyb* gene comprising the sequence of nucleotides set forth in SEQ ID NO: 1 or the rice *GAMyb* gene comprising the sequence of nucleotides set forth in SEQ ID NO: 3.

In the context of the present invention, an antisense molecule is an RNA molecule which is transcribed from the complementary strand of a nuclear gene to that which is normally transcribed to produce a "sense" mRNA molecule capable of being translated into a MYB polypeptide. The antisense molecule is therefore complementary to the sense mRNA, or a part thereof. Although not limiting the mode of action of the antisense molecules of the present invention to any specific mechanism, the antisense RNA molecule possesses the capacity to form a double-stranded mRNA by base pairing with the sense mRNA, which may prevent, delay or otherwise reduce translation of the sense MRNA and subsequent synthesis of a polypeptide gene product. Preferably, the antisense molecule of the present invention targets a barley or rice *GAMyb* mRNA molecule as hereinbefore defined.

In a particularly preferred embodiment, the present invention provides an antisense molecule comprising the 3′ end of *Os*GAMyb cDNA sequence set forth in SEQ ID NO:3 or a complementary strand, homologue, analogue or derivative thereof which is useful

in reducing the expression of a *GAMyb* gene in a monocotyledonous plant species selected from the list comprising barley, wheat, rye, rice, maize or sorghum, amongst others. In a most preferred embodiment, the antisense molecule is capable of reducing expression of the *OsGAMyb* gene (i.e. the rice *GAMyb* gene) in transgenic rice plants expressing the antisense molecule.

Preferably, the reduction in expression of the *GAMyb* gene is at least 10%, more preferably at least 20%, even more preferably at least 50% and even still more preferably at least 75% and yet even still more preferably at least 90%, compared to the expression of the gene in isogenic non-transformed plants or other plants belonging to the same species.

Ribozymes are synthetic RNA molecules which comprise a hybridising region complementary to two regions, each of at least 5 contiguous nucleotide bases in the target sense mRNA. In addition, ribozymes possess highly specific endoribonuclease activity, which autocatalytically cleaves the target sense MRNA. A complete description of the function of ribozymes is presented by Haseloff and Gerlach (1988) and contained in International Patent Application No. WO89/05852. The present invention extends to ribozyme which target a sense mRNA encoding a gibberellin-regulated MYB polypeptide, thereby hybridising to said sense mRNA and cleaving it, such that it is no longer capable of being translated to synthesise a functional polypeptide product. Preferably, the ribozyme molecule of the present invention targets a barley *GAMyb* mRNA molecule.

According to this embodiment, the present invention provides a ribozyme or antisense molecule comprising at least 5 contiguous nucleotide bases which are able to form a hydrogen-bonded complex with a sense mRNA encoding a gibberellin-regulated MYB polypeptide, to reduce translation of said mRNA. Although the preferred antisense and/or ribozyme molecules hybridise to at least about 10 to 20 nucleotides of the target molecule, the present invention extends to molecules capable of hybridising to at least about 50-100 nucleotide bases in length, or a molecule capable of hybridising to a full-

length or substantially full-length gibberellin-regulated *Myb* mRNA.

In a particularly preferred embodiment, the antisense molecule is capable of hybridising to approximately 1000-1500 nucleotides of the target mRNA molecule.

It is understood in the art that certain modifications, including nucleotide substitutions amongst others, may be made to the antisense and/or ribozyme molecules of the present invention, without destroying the efficacy of said molecules in inhibiting the expression of a gene encoding a gibberellin-regulated MYB polypeptide. It is therefore within the scope of the present invention to include any nucleotide sequence variants, homologues, analogues, or fragments of the said gene encoding same, the only requirement being that said nucleotide sequence variant, when transcribed, produces an antisense and/or ribozyme molecule which is capable of hybridising to the said sense mRNA molecule.

Gene targeting is the replacement of an endogenous gene sequence within a cell by a related DNA sequence to which it hybridises, thereby altering the form and/or function of the endogenous gene and the subsequent phenotype of the cell. According to this embodiment, at least a part of the DNA sequence defined by SEQ ID NO:1 or SEQ ID NO:3, or a related gibberellin-regulated *Myb* genetic sequence, may be introduced into target cells containing an endogenous gibberellin-regulated *Myb* gene to replace said genetic sequence, thereby altering said gibberellin-regulated *Myb* gene.

Furthermore, the polypeptide product of said gibberellin-regulated *Myb* genetic sequence possesses different DNA binding affinity/specificity and/or transcriptional activating activity and/or expression characteristics, producing in turn modified expression of genes regulated by said polypeptide, preferably genes involved in the malting process(es), for example α-amylase in particular high pI α-amylase and low pI α-amylase, β-glucanase in particular EII-(1-3,1-4)-β-glucanase, proteases in particular Cathepsin β-like proteases, α-glucosidase, xylanase, arabinofuranosidase, amongst others.

The present invention extends to genetic constructs designed to facilitate expression of

a gibberellin-regulated *Myb* genetic sequence which is identical, or complementary to the sequence set forth in SEQ ID NO:1 or SEQ ID NO:3, or a functional derivative, part, homologue, or analogue thereof, or a genetic construct designed to facilitate expression of a sense molecule, an antisense molecule, ribozyme molecule, co-suppression molecule, or gene targeting molecule containing said genetic sequence.

The said genetic construct of the present invention comprises the foregoing sense, antisense, or ribozyme, or co-suppression nucleic acid molecule, or gene-targeting molecule encoding or complementary to a nucleic acid molecule encoding, a gibberellin-regulated MYB polypeptide, placed operably under the control of a promoter sequence capable of regulating the expression of the said nucleic acid molecule in a eukaryotic cell, preferably a plant cell. The said genetic construct optionally comprises, in addition to a promoter and sense, or antisense, or ribozyme, or co-suppression, or gene-targeting nucleic acid molecule, a terminator sequence.

The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. Terminators are 3'-non-translated DNA sequences containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3'-end of a primary transcript. Terminators active in plant cells are known and described in the literature. They may be isolated from bacteria, fungi, viruses, animals and/or plants. Examples of terminators particularly suitable for use in the genetic constructs of the present invention include the nopaline synthase (NOS) gene terminator of *Agrobacterium tumefaciens,* the terminator of the Cauliflower mosaic virus (CaMV) 35S gene, and the *zein* gene terminator from *Zea mays*.

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a classical genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or

external stimuli, or in a tissue-specific manner. A promoter is usually, but not necessarily, positioned upstream or 5', of a structural gene, the expression of which it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the gene.

In the present context, the term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of said antisense, or ribozyme, or co-suppression nucleic acid molecule, in a plant cell. Preferred promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression of the antisense or ribozyme or co-suppression molecule and/or to alter the spatial expression and/or temporal expression of said sense or antisense, or ribozyme, or co-suppression, or gene-targeting molecule. For example, regulatory elements which confer copper inducibility may be placed adjacent to a heterologous promoter sequence driving expression of a sense, or antisense, or ribozyme, or co-suppression, or gene-targeting molecule, thereby conferring copper inducibility on the expression of said molecule.

Placing a sense or ribozyme, or antisense, or co-suppression, or gene-targeting molecule under the regulatory control of a promoter sequence means positioning the said molecule such that expression is controlled by the promoter sequence. Promoters are generally positioned 5' (upstream) to the genes that they control. In the construction of heterologous promoter/structural gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting, i.e., the gene from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting, i.e., the genes from which it is derived. Again, as is known in the art, some variation in this distance can also occur.

Examples of promoters suitable for use in genetic constructs of the present invention include viral, fungal, bacterial, animal and plant derived promoters capable of functioning in plant cells. The promoter may regulate the expression of the said molecule constitutively, or differentially with respect to the tissue in which expression occurs or, with respect to the developmental stage at which expression occurs, or in response to external stimuli such as physiological stresses, or plant pathogens, or metal ions, amongst others. Preferably, the promoter is capable of regulating expression of a sense, or ribozyme, or antisense, or co-suppression molecule or gene targeting, in a plant cell. Examples of preferred promoters include the CaMV 35S promoter, NOS promoter, octopine synthase (OCS) promoter rice *Actin1* gene promoter and the like. The promoter may also be derived from a genomic clone encoding a gibberellin-regulated MYB polypeptide, preferably the barley or rice *GAMyb* genes.

In a most preferred embodiment, however, the promoter is capable of expression in a monocotyledonous plant cell, for example maize, wheat, barley, rice, sorghum, barley and rye, amongst others.

Particularly preferred promoters according to the present invention include, but are not limited to the rice *Actin*1 promoter, CaMV 35S promoter, *Ubiquitin*1 promoter (Ubi1), *α-amylase* promoter or *EII-(1-3,1-4)-β-glucanase* promoter sequences.

According to this embodiment, one aspect is directed to a genetic construct comprising a promoter or functional derivative, part fragment, homologue, or analogue thereof, from a genomic clone equivalent of the nucleotide sequence defined by SEQ ID NO:1 or SEQ ID NO:3 or a homologue, analogue or derivative therof.

The genetic constructs of the present invention are particularly useful in the production of crop plants with enhanced malting characteristics. Such enhanced malting characteristics are selected from the list comprising, but not limited to, the modulation of dormancy, more uniform germination of seed, high post-kilning levels of hydrolytic

enzymes required during malting, more rapid filtration of the mash, less cloudy precipitate formation and altered alcohol content in the finished product. In a preferred embodiment, the said crop plant is a monocotyledonous plant species selected from the group including wheat, barley, rice, rye, maize, or sorghum. In a particularly preferred embodiment, the crop plant is barley.

The recombinant DNA molecule carrying the sense, or antisense, or ribozyme or co-suppression molecule of the present invention and/or genetic construct comprising the same, may be introduced into plant tissue, thereby producing a "transgenic plant", by various techniques known to those skilled in the art. The technique used for a given plant species or specific type of plant tissue depends on the known successful techniques. Means for introducing recombinant DNA into plant tissue include, but are not limited to, transformation (Paszkowski *et al.*, 1984), electroporation (Fromm *et al.*, 1985), or micro injection of the DNA (Crossway *et al.*, 1986), or T-DNA-mediated transfer from *Agrobacterium* to the plant tissue. Representative T-DNA vector systems are described in the following references: An *et al.*(1985); Herrera-Estrella *et al.* (1983a,b); Herrera-Estrella *et al.* (1985). Once introduced into the plant tissue, the expression of the introduced gene may be assayed in a transient expression system, or it may be determined after selection for stable integration within the plant genome. Techniques are known for the *in vitro* culture of plant tissue, and in a number of cases, for regeneration into whole plants. Procedures for transferring the introduced gene from the originally transformed plant into commercially useful cultivars are known to those skilled in the art.

A still further aspect of the present invention extends to a transgenic plant such as a crop plant, carrying the foregoing sense, or antisense, or ribozyme, or co-suppression, or gene-targeting molecule and/or genetic constructs comprising the same. Preferably, the transgenic plant is one or more of the following: wheat, barley, rice, rye, maize, or sorghum, amongst others. Additional species are not excluded.

In a particularly preferred embodiment, the present invention provides a transgenic rice plant transformed with a *GAMyb* genetic sequence set forth in SEQ ID NO:1 or SEQ ID

NO:3 or a complementary sequence, homologue, analogue or derivative thereof.

In a most particularly preferred embodiment, the transgenic rice plant according to the invention is transformed with an antisense genetic construct comprising a sequence of nucleotides derived from the 3'region of SEQ ID NO:3 or a complementary strand, homologue, analogue or derivative thereof. In an exemplification of this embodiment, there is provided a transgenic rice plant, transformed with an antisense genetic construct comprising nucleotide residues 1003 to 2113 of SEQ ID NO:3.

The present invention further extends to the progeny of a transgenic plant according to any one of the foregoing embodiments.

The present invention is further described by reference to the following non-limiting Figures and Examples.

In the Figures:

Figure 1 is a photographic representation of a Southern blot hybridisation of barley genomic DNA digested with *Bgl*II (lane 1), *Hin*dIII (lane 2), or *Xba*I (lane 3), and probe comprising nucleotides 1430 to 2189, of the nucleotide sequence set forth in SEQ ID NO:1.

Figure 2 shows the effect of GA_3 on *GAMyb* and *α-amylase* gene expression. Panel (A) is a photographic representation of a northern blot hybridisation experiment in which isolated barley aleurone layers were incubated with or without GA_3, RNA was then isolated and probed with a 3' gene-specific *GAMyb* cDNA probe, or a high-pI α-amylase cDNA, as indicated on the Figure. Numbers above the lanes indicate hours after the start of hormone treatment. Panel (B) is a graphical representation showing the time course for induction of expression of the *GAMyb* gene, by application of GA_3.

Figure 3 is a photographic representation of a northern blot hybridisation experiment,

showing the combined effect of GA_3 and ABA on *GAMyb* gene expression. Isolated barley aleurone layers were incubated either without hormone (lane 1), or in the presence of GA_3 (lane 2), ABA (lane 3), or GA_3 +ABA (lane 4), prior to extraction of RNA. Northern blots were probed with either a gene-specific *GAMyb* probe (top panel), or as a control, with the α-amylase probe, as described in the legend to Figure 2.

Figure 4 is a photographic representation of a northern blot hybridisation experiment showing the differential effect of cycloheximide on *GAMyb* and *α-amylase* gene expression levels. Isolated aleurone layers were incubated for 6 hr, with either no GA_3 or cycloheximide (lane 1), cycloheximide alone (lane 2), GA_3 alone (lane 4), or GA_3 plus cycloheximide (lane 4). RNA was then isolated and probed with the probes described in the legend to Figure 2.

Figure 5 is a diagrammatic representation showing the effector constructs pAct1.*GAMyb* and pAct1.*C1* and the reporter constructs Am(-174)IGN and mTAACAAA-1.

Figure 6 shows binding of the GAMYB protein to a 22 basepair α-amylase promoter fragment containing the TAACAAA box. Panel (A) is a diagrammatic representation showing nucleotide sequences of oligonucleotide probes used in gel mobility shift assays. Probe w contains wild-type α-amylase promoter sequence, from -149 to -128 (Jacobsen and Close, 1991). Probes m1-5 contain mutations in the TAACAAA box, as indicated. Panel (B) is a photographic representation showing a gel mobility shift assay using affinity-purified recombinant GAMYB protein and ^{32}P-labelled oligonucleotide probes described in panel (A). Lane 1 contained probe w without protein; lane 2 , probe w and recombinant glutathione-S-transferase (GST); lane 3, probe w and GAMYB; lane 4, probe m-1 and GAMYB; lane 5, probe m-2 and GAMYB; lane 6, probe m-3 and GAMYB; lane 7, probe m-4 and GAMYB; lane 8, probe m-5 and GAMYB.

Figure 7 is a photographic representation of a gel mobility shift assay showing competition for binding of recombinant GAMYB protein to probe w. Unlabelled competitor oligonucleotides w (lanes 1-3), m-1 (lanes 4-6), m-2 (lanes 7-9), m-3 (lanes

10-12), m-4 (lanes 13-15), and m-5 (lanes 16-18), were incubated with affinity-purified recombinant GAMYB protein, prior to the addition of labelled probe w. The competitor DNA used is indicated at the top of each gel in the figure. In each gel indicated in the figure, the left-hand lane (i.e. lanes 1,4,7,10,13,16) shows the DNA-protein complex formed in the absence of any competitor DNA. The middle lane in each gel (i.e. lanes 2,5,8,11,14,17) shows the effect of adding a 10-fold molar excess of each competitor DNA. The right-hand lane in each gel (i.e. lanes 3,6,9,12,15,18) shows the effect of adding a 100-fold molar excess of each competitor DNA.

Figure 8 shows transient expression analysis of high-pI α-amylase promoter with GAMYB binding-site mutations. Panel (A) is a diagrammatic representation showing wild-type and mutant α-amylase promoters sequences. Am(-174)IGN contains a wild-type binding site for the GAMYB protein. mTAACAAA-2 and -3 contain the single base-pair mutations indicated, in the GAMYB binding site. Panel (B) is a graphical representation showing GA_3-responsiveness of the wild-type and mutant high-pI α-amylase promoters. Am(-174)IGN, mTAACAAA-2 and -3 were bombarded into intact aleurone cells and incubated with either no hormone (control), or GA_3. β-glucuronidase (GUS) enzyme activity was determined. All GUS activity values are shown relative to the activity of Am(-174)IGN. Error bars represent standard error of the mean (n=11).

Figure 9 is a graphical representation showing transactivation of the high-pI α-amylase promoter by the GAMYB protein, in barley aleurone cells. Constructs used are shown in Figure 5. Intact aleurone cells were bombarded with reporter constructs and effector constructs as indicated, and incubated either without hormone (control), or in the presence of GA_3. The error bars represent the standard error of the mean (n=12 to 34).

Figure 10 is a tabular representation showing transactivation of the promoters of three cereal aleurone genes, the barley *Amy* 32b gene promoter, the barley EII-(1-3,1-4)-β-glucanase gene promoter and the wheat Cathepsin β-like gene *Al*21 promoter, in barley aleurone cells. The p113Act1.cas and Act1.*GAMyb* constructs are described in Example 6. Plasmid EII.IGN contains 1600bp of the EII-(1-3,1-4)-β-glucanase gene promoter

placed upstream of a reporter gene cassette comprising maize *Adhl* intron1/GUS/nopaline synthase 3′ terminator sequences (which are also contained in plasmid pAm(-174) IGN described in Example 6). Plasmid mlo22 contains the barley *Amy 32b* promoter linked to a GUS reporter gene and is described by Lanahan *et al.* (1992). Plasmid CBG1 contains the wheat Cathepsin β-like gene promoter *Al*21 linked to a GUS reporter gene and is described by Cejudo *et al.* (1992). Intact barley aleurone cells were bombarded with reporter and effector constructs as indicated, and incubated either without hormone or in the presence of GA_3. In the case of the Dhn7 construct, intact aleurone cells were incubated without hormone (control) or in the presence of ABA. GUS activities were determined after 24 h incubation and are shown relative to the activity of the respective construct co-shot with the Act1.cas control effector construct.

Figure 11 is a schematic representation of the proposed GA response pathway for high-pI α-amylase gene expression in barley aleurone cells.

Figure 12 is a schematic representation of an alignment of the deduced amino acid sequences of rice (upper) and barley (lower) GAMYB polypeptides and. The aligned R2 and R3 repeats of the MYB DNA binding domain are boxed. A putative transcriptional activation domain present in both sequences is underlined.

Figure 13 is a photographic representation of a northern blot hybridisation showing the effect of gibberellin on *GAMyb* and *α-amylase* gene expression in rice endosperm. RNA was isolated from rice endosperm half grains (var Taipai 309) (Schuurink *et al.*, 1996) which had been hydrated in 10 mM $CaCl_2$ overnight at 28°C and then incubated in 10 mM $CaCl_2$, 150 μm ml^{-1} cefotaxime, 50 units ml nystatin, and either no hormone (control) or 10^{-6} M gibberellic acid (GA) at 30°C. After blotting the RNA was probed with a gene-specific 3′ *OsGAMyb* probe (panel a); a low-pI barley α-amylase cDNA (panel b), and a wheat ribosomal DNA clone pTA250 (Gerlack and Bedbrook, 1979; panel c). Numbers above each lane indicate hours (h) after the start of the treatment. 10 μg RNA was loaded per lane.

Figure 14 is a schematic representation showing transactivation of barley low-pI α-amylase promoter by rice GAMYB in barley aleurone cells. (A) The GAMyb effector construct was made by inserting the *OsGAMyb* cDNA containing the entire open reading frame into the multi cloning site in the maize Ubiquitin gene expression cassette, Ubil.cas, comprising the maize Ubiquitin gene promoter fused to a multi cloning site and Nos 3′ terminator. The reporter construct, mlo22 consists of the barley *Amy*32b promoter fused to a GUS reporter gene (Lanahan *et al.*, 1992). (B) Intact aleurone cells were co-bombarded with the reporter construct and effector constructs and incubated with no hormone (control) and gibberellic acid (GA) as described in Examples 10-12. Preparation of extracts and assays of GUS activity were as described by Gubler *et al.* (1995).

Figure 15 is a photographic representation of a western blot analysis of the GAMYB fusion protein. In panel (a), the GAMYB fusion protein was detected by immunoblotting with purified antibody raised against a synthetic peptide comprising amino acids 1-14 of barley GAMYB. GST, glutathione-s-transferase; GST-GAMyb, GST-GAMYB fusion protein; PI, pre-immune sera; I, immune sera. The numbers on the left of the figure indicate apparent molecular mass, in kDa. In panel (b), the GAMYB fusion protein was detected by immunoblotting with purified antibody raised against a synthetic peptide comprising amino acids 481-494 of the barley GAMYB polypeptide . Pre-immune or immune sera (0.1 to 10 μg ml^{-1}) were incubated with blots containing GST-GAMYB fusion protein. The numbers on the left of the panel indicate apparent molecular weight, in kDa.

Figure 16 is a schematic representation of an antisense genetic construct comprising the ubiquitous promoter sequence operably connected to the *OsGAMyb* cDNA sequence placed in antisense orientation upstream of the nopaline synthase terminator sequence.

Figure 17 is a photographic representation of a Southern blot hybridisation demonstrating integration of the Ubil.as*Os*GAmyb construct into the genomic DNA of 6 sibling T_0 rice plant lines (JSH 15.2.28 lines 1-6). The plasmid control (lane marked "1 copy") and the

genomic DNA (lanes 1-6) had been digested with the enzyme *Eco*RI. The blot was probed with 3′ end of the *OsGAMyb* cDNA.

Figure 18 is a graphical representation showing *HvGAMyb* mRNA levels in germinating barley embryos.

Single letter and three letter abbreviations used for amino acid residues in the specification are defined in Table 1.

TABLE 1

Amino Acid	Three-letter Abbreviation	One-letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

EXAMPLE 1

Molecular cloning of *GAMyb* cDNA

A barley *(Hordeum vulgare* cv Himalaya) cDNA library prepared from isolated aleurone layers (Stratagene, La Jolla, California) was screened for putative *Myb* cDNAs. The library was screened with a 174 bp PCR fragment which contained sequences homologous to the region between conserved R2 and R3 repeats of the maize C1 cDNA (amino acid 49 to 106). The probes were labelled [α-^{32}P]dCTP by random priming. Hybridization was in 6 x SSC and 0.1% SDS, 1 x Denhardt's solution, 100 μg/ml salmon sperm DNA at 54°C and washed in 3 x SSC, 0.1% SDS at 54°C (Jackson *et al.*, 1991). Positive plaques were purified by a further round of plaque hybridisation. The pBluescript Sk(-) plasmid containing the *GAMyb* insert was excised *in vivo* according to the manufacturer's instructions (Stratagene). The cloned cDNA fragment (p*GAMyb)* was sequenced by the dideoxy terminator cycle sequencing method using an Applied Biosystems' 370A DNA sequencer. From an initial screen of 10^6 recombinant phages one partial cDNA clone, *GAMyb,* was isolated that contained a 1991bp insert.

Amplification of 5′ end of cDNAs was performed by the 5′ RACE procedure as described by Frohman (1990). Poly (A)+ RNA from GA-treated barley aleurone layers was reverse transcribed using a gene specific primer 5′-TGTTCTTCTGCACCGCGTTC-3′. Following removal of excess primer, a 5′ poly A tail was synthesised on the single-stranded cDNA using terminal deoxynucleotidyl transferase. PCR amplification was performed using a nested 3′ gene-specific primer 5′-GTGCTTCACGTACTCCAC-3′ and an oligo dT primer. PCR fragments were cloned by blunt-end ligating into pCR-Script (Stratagene) according to the manufacturer's instructions. Positive clones were identified by colony hybridisations (Sambrook *et al.*, 1989) and sequenced.

The longest PCR product cloned was 408 bp and it was shown by sequence analysis to extend the cDNA sequence a further 271bp. Partial sequencing of a Himalaya barley genomic clone which contains the *GAMyb* gene confirmed the sequence of the 5′ RACE

clone. The complete nucleotide sequence of the full-length barley *GAMyb* cDNA and deduced amino acid sequence is shown in SEQ ID NO: 1 and SEQ ID NO: 2, respectively.

The longest open reading frame of SEQ ID NO: 1 extends 1659 bp from position 275 to a TAG stop codon found at positions 1934-1936 and encodes a polypeptide of 553 amino acid residues. Amino acid residues 42 - 94 (R2) and 95 - 145 (R3) of SEQ ID NO: 2, are highly homologous to the R2 and R3 regions of the DNA-binding domain of animal and plant MYB proteins. Both these repeats contain conserved tryptophan residues which play a critical role in stabilizing the DNA-binding domain of animal MYBs (Ogata *et al.*, 1992). Outside the putative DNA binding domain, there is little similarity between the derived amino acid sequences of GAMYB and other MYBs.

EXAMPLE 2

Genomic DNA isolation and Southern analysis

DNA was isolated from etiolated leaves from 6 day old barley seedlings using the method described by Dellaporta *et al.* (1993). For detection of *GAMyb* gene in genomic DNA, 25μg of genomic DNA digested with *Bgl*II or *Hin*dIII or *Xba*I was fractionated on 1% (w/v) agarose gel and blotted onto nylon membrane. The blot was hybridized with a ^{32}P-labelled PCR fragment which contained sequences between nucleotides 1430 and 2189 of the nucleotide sequence set forth in SEQ ID NO: 1. Hybridisation was carried out at 42°C in 6 x SSC, 5 x Denhardt's solution [0.1% (w/v) Ficoll, 0.1% (w/v) PVP and 0.1 % (w/v) BSA], 0.1% (w/v) SDS and 50% (v/v) formamide. The blots were washed in 0.1 x SSC, 0.1% (w/v) SDS at 65°C.

Southern analysis of barley genomic DNA cut with *Hin*dIII and *Xba*I using the 3′ end of the cDNA (from position 1430 to 2189) as a probe, showed only one band which hybridized with the probe (Fig. 1). This result indicates there is only one copy of the *GAMyb* gene. When the DNA was cut with *Bgl*II, two bands were observed with the 3′

probe. The appearance of two bands is due to an internal *BglII* site within the sequences that were probed.

EXAMPLE 3

GAMyb gene expression is regulated by GA

Aleurone layers were prepared from grains of *Hordeum vulgare* cv Himalaya (1985 harvest, Washington State University, Pullman) as described previously (Chrispeels and Varner, 1967). The isolated layers were isolated from 3 day imbibed de-embryonated half grains and incubated in flasks containing 2 ml 10 mM $CaCl_2$, 150 μg/ml cefotaxime, 50 IU/ml nystatin and no hormones (control), or 10^{-6}M gibberellic acid (GA) or 10^{-6}M gibberellic acid and 5 x 10^{-5} M abscisic acid (ABA) (GA + ABA) at 25°C for various times. Following hormone treatments, the layers were stored in liquid nitrogen until required. Aleurone layers that were to be treated with cycloheximide, were pre-incubated with 50μM cycloheximide, calcium chloride and antibiotics for 30 min before the hormones were added.

RNA was isolated from aleurone layers according to Chandler and Jacobsen (1991) with one minor modification. Bentonite was omitted from homogenisation medium. For Northern analysis, 20 μg of aleurone RNA was fractionated in 1% (w/v) agarose gel containing formaldehyde and blotted onto nylon membrane. The blots were hybridized with a ^{32}P-labelled 991 bp PCR product containing gene specific *GAMyb* sequences (nucleotide 1198 to 2189 of the sequence set forth in SEQ ID NO: 1). Hybridisation conditions were essentially as described in Example 2.

After autoradiography, Northern blots were stripped of the *GAMyb* probe, and reprobed with a 1.1 kb DNA fragment containing the pHV19 cDNA clone, which is a barley high-pI amylase cDNA isolated from GA_3-treated aleurone layers (Chandler *et al*, 1984) and a control 9 kb wheat rRNA clone, pTA71 (Gerlach and Bedbrook, 1979). All DNA probes were labelled by oligonucleotide priming (Feinberg and Vogelstein, 1983).

mRNA transcripts were quantified using a Molecular Dynamics Phosphorimager and ImageQuant software. *GAMyb* and high-pI α-amylase mRNA transcript levels were normalized to rRNA levels to overcome error in RNA quantitation by spectrophotometry.

To test whether *GAMyb* gene expression was GA-regulated, Northern blots of RNA from control and GA_3-treated barley aleurone layers from various time points were probed using a 3′ gene-specific *GAMyb* cDNA probe (Fig. 2A). In control treatments, *GAMyb* mRNA levels remained low for the first 12 h and then increased slightly over the next 12 h. In contrast, *GAMyb* mRNA levels increased rapidly in response to GA_3. By 12 h, the level in GA_3-treated aleurone layers was approximately 5 fold higher than that found at the corresponding time for control treatments. However the increase in mRNA levels was transient. Between 12 and 24 h, the level of mRNA had declined by 50%. The same blot was stripped and hybridized using the barley high-pI α-amylase cDNA clone, PHV19 (Chandler *et al.* 1984). The α-amylase mRNA was found to be strongly induced by GA_3.

To compare the kinetics of *GAMyb* and α-amylase mRNA accumulation in GA_3-treated barley aleurone layers, transcript levels in the blot shown in Fig 2a were quantified using a phosphorimager and normalized to rRNA. The time course shown in Fig. 2B shows that the expression of the *GAMyb* gene precedes α-amylase gene expression in GA_2-treated aleurone layers. *GAMyb* mRNA levels rise dramatically within the first 3 h after GA_3 addition, while the maximum rate of increase in α-amylase mRNA levels occurs later between 6 to 12 h. Examination of earlier time points indicted that *GAMyb* mRNA levels increased within 1 hr in response to GA_3. Maximal *GAMyb* mRNA levels (6-12 h) coincided with the maximal rate of accumulation of α-amylase mRNA indicating the possibility that the expression of the two genes may be causally linked.

EXAMPLE 4

GAMyb gene expression is regulated by abscisic acid

Abscisic acid (ABA) has been shown to antagonize GA action in barley aleurone layers. To test whether GA_3-induced *GAMyb* gene expression is also down-regulated by ABA, we probed Northern blots of RNA from control-, GA_3-, ABA- and GA_3 + ABA-treated barley aleurone layers as described in Example 3 (Fig 3). The response of *GAMyb* gene expression to GA_3 and ABA was very similar to that observed for α-amylase gene expression. Control and ABA-treated layers had very low levels of *GAMyb* and α-amylase mRNA. Quantification of mRNA levels using the phosphoimager indicated that the GA_3-induced increases in *GAMyb* and α-amylase mRNA levels was inhibited by ABA by about 40-50% and 80-100%, respectively.

To test whether the differential response of *GAMyb* and α-amylase gene expression to ABA was due to slowness of aleurone cells to respond to ABA to take up ABA, we preincubated aleurone layers with ABA for 30 min before adding GA_3. The ABA preincubation had no effect on *GAMyb* gene expression compared to treatments where there was no preincubation. The differential response indicates that α-amylase gene expression is far more responsive to ABA that *GAMyb* gene expression in barley aleurone.

Data presented in Examples 3 and 4 open up new insights into the mechanisms of ABA action in aleurone cells. The inhibition of α-amylase gene expression by ABA may be explained at least in part by the action of ABA on *GAMyb* gene expression. ABA inhibited GA-induced increases in *GAMyb* transcript levels by up to 50%. However ABA caused a far greater decrease in steady state levels of α-amylase mRNA than *GAMyb* mRNA indicating that the regulation of GAMYB activity may not be all at the transcriptional level. It is possible that ABA action regulates GAMYB activity not only at the level of transcription but at the level of post-transcription by promoting GAMYB phosphorylation. ABA may also be regulating the expression of MYBs which compete

for the GAMYB-binding site in the α-amylase promoter. It is of interest to speculate that the known antagonistic effect of these two hormones at the level of gene expression may involve, in part, the expression of mutually antagonistic MYBs which are under the control of ABA or GA.

EXAMPLE 5

Cycloheximide induces the expression of *GAMyb*

To test whether *GAMyb* gene expression is also sensitive to the protein synthesis inhibitor, cycloheximide, we performed Northern blot analysis using a 3′ gene specific *GAMyb* probe and a high-pI amylase cDNA probe (Fig. 4). The *GAMyb* mRNA levels in barley aleurone layers increased in response to GA_3 and cycloheximide alone, but the increase was much greater in the cycloheximide- treated layers compared to GA_3-treated layers. When the tissue was incubated with both GA_3 and cycloheximide, the increase in *GAMyb* mRNA was greater than with either treatment alone.

These results suggest strongly that protein synthesis is not required for GA induction of *GAMyb* gene expression and therefore the *GAMyb* gene is the first gene to be expressed in the GA-response pathway leading to expression of the high-pI α-amylase gene. The mechanisms involved in the induction and super-induction of mRNAs by cycloheximide are not yet clearly understood but may be due to mRNA stabilization.

EXAMPLE 6

Plasmid Construction

The construct Am(-174)IGN, which has been previously described (Jacobsen and Close, 1991), contains a deletion of a high-pI α-amylase gene promoter from barley (-174 to +54) fused to a reporter gene cassette (maize *Adh1* intron 1/GUS/nopaline synthase 3′ terminator sequence). The mTAACAAA-2 and mTAACAAA-3 constructs were created

by using PCR to introduce single base pair mutations within the TAACAAA box of the Am(-174)IGN construct. Mutagenic forward primers are as follows:
mTAA11 (5'-GCCTGCAGGTCGACTCTAGAGAATCGCCTTTTGAGCTCA
CCGTACCGGCCGATGACAAACTCCGG-3");
mTAA12 (5'-GCCTGCAGGTCGACTCTAGAGAATCGCCTTTTGAGCTCA
CCGTAQCCGGCCGATAACAGACTCCGG-3");
reverse primer Am-41 (5'-GTGTGCTGCGCAGCATGCCGG-3").

PCR was performed using the mutagenic forward primers and the reverse primers, and pAm(-174)IGN as template DNA. The PCR products were cut with *Pst*I and cloned into the *Pst*I site at -41 in pAm(-41)IGN (Jacobsen and Close, 1991) thus producing Am(-174)IGN constructs containing single base-pair mutations at -142 (TGACAAA) and at -138(TAACAGA).

The pGEX vector (Smith and Johnson, 1988) was used for the production of glutathione S-transferase fusion proteins for gel mobility shift experiments. The pGEX.*GAMyb* construct was made by cloning a 1656-bp EcoRI fragment containing the *GAMyb* gene coding region (nucleotides 275 to 1930 of SEQ ID NO:1) into the pGEX vector.

GAMyb and *C1* effector constructs used in transient over expression experiments were synthesised by cloning fragments containing the *GAMyb* or *C1* coding regions into the multi cloning site of a plant expression vector, p113Act1.cas, containing the rice *Actin1* promoter and 5' untranslated leader sequence (including intron 1) fused to a multi cloning site-Nos 3' terminator. The pAct1.*GAMyb* construct used in over expression experiments was prepared by first cloning the above-identified *Eco*R1 *GAMyb* fragment which contains the coding region into the *Eco*R1 site of pBluescript SK$^-$vector (Stratagene). The entire *Eco*R1 insert was then cut out as a *Hind*III-*Xba*I fragment and cloned directly into the multicloning site of the p113Act1.cas. The pAct1.*C1* construct which contains the entire coding region of the *C1* cDNA cloned downstream of the rice *Actin1* promoter was also prepared using a similar strategy.

EXAMPLE 7

Production and Purification of GAMYB fusion protein

Purified proteins for gel-mobility shift assays were prepared from *Escherichia coli* XL1-blue cells, transformed with the pGEX.*GAMyb* construct prepared as described in the foregoing Example 6. The transformed cells were grown in 500 ml 2 x YT medium containing 100 μg/ml ampicillin and 1 mM isopropyl β-D-thiogalactoside for 6 h at 25°C, to induce the production of glutathione-S-transferase fusion proteins. The cells were harvested by centrifugation and resuspended in lysis buffer (10mM Tris-HCl (pH 8.0), 0.4 M NaCl, 5 mM $MgCl_2$, 5% (v/v) glycerol, 0.5 mM EDTA). The cells were centrifuged again and the pellet resuspended in lysis buffer. The cells were lysed by a cycle of freezing and thawing followed by four 10 sec bursts of sonication. Triton X-100 and PMSF was added to the lysed cells to the concentration of 1% (v/v) and 100 μg/ml, respectively. The lysed preparation was centrifuged and the supernatant was mixed with pre-swollen glutathione Sepharose-4B beads and incubated at 20°C for 2 min. After absorption, the beads were spun down, washed three times with 50 ml of 75 mM Hepes-KOH (pH 7.9) and 150 mM NaCl. The fusion protein were eluted by washing the beads in 200 μl of 75 mM Hepes-KOH, pH 7.9, 150 mM NaCl and 5 mM of reduced glutathione. Glycerol was added to the eluted protein to a final concentration of 10% (v/v) and aliquots were snap frozen in liquid nitrogen and stored at -80°C.

EXAMPLE 8

Gel mobility shift assays

Complementary oligonucleotides with 5′ overhangs containing native and mutant sequences of the high-pI amylase promoter (-149 to -128) were synthesised: The nucleotide sequences of the oligonucleotides used are shown in Table 2.

TABLE 2: Oligonucleotides used in gel retardation assays

Oligonucleotide	Nucleotide sequence*
w	5'-GGCCGATAACAAACTCCGG-3'
m-1	5'-GGCCGACTCGAGACTCCGG-3'
m-2	5'GGCCGATGACAAACTCCGG-3'
m-3	5'-GGCCGATAACAGACTCCGG-3'
m-4	5'-GGCCGATAACAAGCTCCGG-3'
m-5	5'-GGCCGATAACAAAATCCGG-3'

*Only sequences of upper strands are shown. Lower strands are complementary sequences. Mutated nucleotide residues, relative to the wild-type sequence, are indicated in bold typeface.

After annealing the complementary oligonucleotides were 3' end-labelled using ^{32}P-dCTP and the Klenow fragment, and purified by electrophoresis on polyacrylamide gels. Bacterially-produced GAMYB fusion protein-oligonucleotide binding reactions were performed in 10 μl binding buffer (24 mM Hepes-KOH pH 7.9, 50 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10% (v/v) glycerol) with 2 μg of poly(dI-dC)-poly(dI-dC), 150 ng of GAMYB fusion protein and 0.9 ng of a ^{32}P-labelled oligonucleotide probe (40,000cpm). After incubation at 20°C for 10 min, the samples were run on a 6% (w/v) polyacrylamide gel containing 5% (v/v) glycerol, in 0.25 x Tris-borate-EDTA buffer at 140 volts. After electrophoresis, the gels were dried and autoradiographed.

EXAMPLE 9

GAMyb binds to the α-amylase promoter

To test whether GAMYB binds to the TAACAAA box in the high-pI α-amylase promoter, we performed gel-retardation experiments as described in Example 8. We looked for binding between an oligonucleotide probe (w) containing the amylase promoter sequence (-149 to -128), including the wild-type TAACAAA box and an affinity-purified glutathione-s-transferase (GST)-GAMYB fusion protein that had been expressed in *Escherichia coli* according to Example 7. As shown in Figure 6, the GST-GAMYB fusion protein bound to probe w, resulting in the formation of a retarded complex (lane 3). In contrast, the GST protein alone failed to bind (lane 2). Cold competition with 100 fold molar excess of the unlabelled w fragment, completely abolished complex formation between the w probe and GAMYB fusion protein, indicating that binding is reversible. Experiments were also performed using mutant oligonucleotide probes (m-1 to m-5). As shown in Figure 6, GAMYB only bound to the m-3 probe, confirming that GAMYB can bind to the mutated sequence TAACAGAc, which resembles the TAACAAA box counterpart, in barley low-pI α-amylase promoters.

To determine the binding site of the *GAMyb* within the 22 bp w probe, we used a series of mutant probes containing single (m-2 to m-5) and multi(m-1) base pair mutations as unlabelled competitors for binding to the wild-type (w) probe. Extensive mutation of the TAACAAA box of CTCGAGA in the m-1 probe, abolished binding of the GAMYB (Fig. 7a, lane 4) indicating that the GAMYB binding site includes at least part of the TAACAAA box. A similar mutation of the TAACAAA box in the α-amylase promoter had been previously shown to cause a decrease in GA-induced expression in transient expression analyses (Gubler and Jacobsen, 1992).

As shown in Figure 7b, GAMYB fails to bind m-2 (lane 5) which contains a single base pair mutation (TGACAAAc) in the AAC core of the 5′ putative MYB binding site. In contrast, a similar mutation in the 3′ putative binding site (AGActccg) had no effect on

GAMYB binding (Figure 7b, lane 6). These results indicate that the GAMYB binds to the 5′ MYB binding site, TAACAAAc and not the 3′ site. Further single mutations of the GAMYB binding site, TAACAAAc were introduced to confirm the 3′ end of the binding site. GAMYB failed to bind to m-4 and m-5 (Figure 7b, lanes 7 and 8) which carry single mutations at the 3′ end of the hexameric binding site.

EXAMPLE 10

Transient expression analyses

Barley Himalaya half-seeds were prepared for particle bombardment as described by Lanahan *et al.*, (1992). Plasmid constructs used for transient expression analyses were as described in Example 6. Plasmid DNA purified on Qiagen-tips (Qiagen, Hilden, Germany) was coated onto 1.6 μm gold particles, essentially as described by Hunold *et al.* (1994). For expression analysis of mutant α-amylase promoter constructs, 0.5 μg Am(-174)IGN, or mTAACAAA-2, or mTAACAAA-3 constructs were precipitated onto 0.75 mg of gold. For experiments involving *GAMyb* gene overexpression, 1.5 μg of effector constructs (pAct1.*GAMyb* or pAct1.*C1*) and 0.5 μg of reporter constructs [Am(-174)IGN or mTAACAAA] were precipitated onto 0.75 mg of gold. An internal standard (ubiquitin promoter-luciferase reporter construct) was not used in these experiments because of difficulties ascertaining whether overexpression of *GAMyb* or *C1* had any effect on the expression of the internal standard. Each experiment was highly-replicated ($n > 12$). The conditions used for bombardment using a helium particle inflow gun (Finer *et al.*, 1992) were the same as those described in Gubler *et al.* (1995) but with one modification. Six barley half grain were used per shot, instead of eight. After shooting, the six half seeds were cut longitudinally along the groove resulting in two equal quarter-grains which were then distributed into flasks containing 10 mM $CaCl_2$ (control) or 10 mM $CaCl_2$ and 1 μm GA_3. Both incubation solutions contained cefotaxime and nystatin, at the same concentrations as described in Example 3. After incubation for 24 h at 25°C, the shot grains were frozen and stored at -70°C. Soluble protein extracts were prepared and assayed for GUS enzyme activity as described previously (Gubler *et al.*, 1995).

The GAMYB binding site, TAACAAAc, has been shown to be functional in transient expression assays. The clustered point mutations introduced in the m-1 sequence, which abolished GAMYB-binding, has previously been shown to greatly reduce the GA-responsiveness of the high-pI α-amylase promoter in transient expression experiments (Gubler and Jacobsen, 1992). To further test whether the GAMYB binding site is functionally important in conferring GA-responsiveness to the α-amylase promoter, mutations used in the binding studies were introduced into the construct Am(-174)IGN and the mutant promoter constructs were analysed by transient expression. As shown in Figure 8, the introduction of a single base pair mutation, TGACAAAc, which abolished GAMYB-binding (Fig. 7), strongly reduced GA-regulated expression. In contrast, the mutation TAACAGAc had no effect on reporter gene expression or GAMYB binding. These results indicate that there is a close correlation between GAMYB binding site and sequences which are functionally important in the GA-response.

EXAMPLE 11

The GAMYB polypeptide is a transcriptional activator of a high-pI α-amylase gene promoter

To test the *in vivo* function of the protein encoded by the *GAMyb* cDNA, we determined whether GAMYB could transactivate a high-pI α-amylase gene promoter:GUS construct. Barley aleurone tissue was co-shot with a GUS reporter gene fused to an high-pI α-amylase promoter [Am(-174)IGN] and a *GAMyb* effector plasmid as described in preceding Example 10 (Fig 5). The effector plasmid consisted of a rice *actin1* promoter fused to the *GAMyb* cDNA. Fig 9 shows that reporter GUS activity which is normally induced by GA_3 was also induced in the absence of GA by the *GAMyb* effector construct. The increase in GUS activity in response to *GAMyb* expression was similar to that found with GA_3 alone. Mutation of the TAACAAA box in the α-amylase promoter (as in the m-1 sequence in Table 2) greatly reduced the response of the reporter construct to GA_3 and the *GAMyb* effector construct. These results indicate that GAMYB is a transcriptional activator which activates high-pI α-amylase gene expression in barley

aleurone cells. In addition, the results provide functional evidence for the GAMYB transcription factor activating expression *via* the TAACAAA box, a binding site for this transcription factor.

To test whether other plant MYBs can activate the α-amylase promoter, the maize *C1* cDNA was fused to the rice *actin1* promoter (Fig. 5) and co-shot with Am(-174)IGN. In transient expression analyses (Fig. 9), the maize *C1* cDNA failed to transactivate the reporter gene. Furthermore, expression of the *C1* cDNA partially inhibited the GA_3 response.

EXAMPLE 12

The GAMYB polypeptide is a transcriptional activator of a number of GA-responsive gene promoters

To test whether GAMYB could transactivate promoters of other genes which are expressed in aleurone cells in response to GA_3, we co-bombarded aleurone cells with reporter constructs containing GUS genes fused to the promoters of the barley *Amy32b* gene (mlo22; described in Lanahan *et al.*, 1992), the barley EII-(1-3,1-4)-β-glucanase gene (EII.IGN; the construct contains 1600 bp of the promoter fused to the reporter gene cassette IGN), and the wheat Cathepsin β-like gene, *Al21* (CBG1, described in Cejudo *et al.*, 1992). As a control, we also tested whether GAMYB could transactivate an ABA-responsive gene promoter *Dhn7* fused to the reporter gene cassette IGN [*Dhn7*(-935).IGN, described in Robertson *et al.*, 1995). The effector plasmid consisted of the rice *actin1* expression cassette p113 Act1.cas (Example 6) with and without the *GAmyb* cDNA.

Figure 10 shows that reporter activity driven by the *Amy 32b*, *EII* and *Al21* gene promoters were induced by the GAMYB effector construct in the absence of GA_3. The increase in GUS activity in response to GAMYB expression was higher than that found with GA_3 alone for all constructs. GAMYB only weakly transactivated the *Dhn7* gene

promoter. These results indicate that GAMYB is a transcriptional activator not only of α-amylase genes but other GA-responsive genes expressed in cereal alaurone. These include the EII-glucanase gene and a protease gene, *Al21*.

EXAMPLE 13

A model of the GA response signal transduction pathway

While not being bound by any theory, or mode of action, these data suggest that *GAMyb* gene expression is required for the expression of genes encoding malting enzymes, for example α-amylase, amongst others. In fact, it is likely that the GAMYB polypeptide is required for the expression of genes encoding all malting enzymes and acts as a "master switch" in the regulation of these genes. The preceding examples indicate that *GAMyb* gene expression in barley aleurone layers, is induced by gibberellin. Comparison of the kinetics of expression indicates that *GAMyb* expression is an early event, in the GA response pathway, and precedes α-amylase expression. Our results provide three lines of evidence to support this proposal. Firstly, we showed that GAMYB fusion protein binds to a GARC *cis*-acting element, the TAACAAA box, in a barley high-pI α-amylase gene promoter (Example 9). By introducing mutations into the TAACAAA box and adjoining 3′ sequence, the binding site was mapped to include the sequence TAACAAAc. Secondly, there is a strong correlation between the GAMYB binding site as defined *in vitro* and the sequences which are functionally important in transient expression assays (compare Example 9 and Example 10). We showed that oligonucleotides containing multiple or single point mutations in the TAACAAAc binding site that abolish binding to GAMYB fusion protein also reduced the GA-responsiveness of the high-pI α-amylase promoter. This provides strong evidence that the GAMYB protein functions *in vivo* to activate α-amylase gene expression through the TAACAAA box. Thirdly, we demonstrated in transient expression experiments that GAMYB activates transcription of several GA-responsive genes expressed in cereal aleurone, including the activation of a high-pI α-amylase promoter, the barley *Amy32b* promoter, the barley EII-(1-3,1-4)-β-glucanase promoter and the wheat *Al21* promoter fused to a

GUS reporter gene in the absence of GA_3 (Examples 11 and 12). Mutation of the TAACAAA box greatly reduced the ability of GAMYB to transactivate the α-amylase promoter construct, confirming that the TAACAAA box is the site of GAMYB binding and transactivation.

Fig 11 shows a model of the GA response pathway between the expression of the high-pI α-amylase gene and the initial GA signal, based on our results. In this model, GA binds to a receptor, presumably on the plasmamembrane and activates a signal transduction pathway which triggers *GAMyb* gene expression. The newly synthesised GAMYB protein in its turn activates the expression of the high-pI α-amylase gene. Our results indicate that the GAMYB is the sole GA-regulated transcription factor required for transcriptional activation of the high-pI α-amylase gene. In the absence of GA, we were able to activate high-pI α-amylase gene transcription by transiently expressing *GAMyb*, under the control of a constitutive promoter. Other transcription factors which are presumably necessary for high-pI α-amylase gene expression (eg factors which may bind to the pyrimidine and TATTCCAC boxes) are likely to be present in aleurone cells that have not been treated with GA.

The wide occurrence of TAACAAA box like sequences in promoters of other genes which are expressed in cereal aleurone cells in response to GA (Huang *et al.*, 1990) may indicate a broad action of the GAMYB protein. Detection of a nuclear factor from barley aleurone layers which binds to TAACAGA box and associated 3′ sequences a barley low-pI α-amylase promoter in a GA-dependent manner (Sutliff *et al.*, 1993) is consistent with our model shown in Fig 11. It seems probably that the binding factor is likely to be GAMYB or a GAMYB complex, since GAMYB was shown in this present study to bind to m3 probe which contains the sequence TAACAGA. Other GA-responsive gene promoters also contain sequences which resemble the GAMYB-binding site in the barley high-pI α-amylase promoter. Deletion analysis in the 5′ region of the barley EII (1-3,1-4)-β-glucanase gene promoter (Wolf, 1992) and wheat cathepsin B-like gene promoter (Cejudo *et al.*, 1992) showed that the GA-responsive regions were downstream of -310 and -173, respectively. The sequence between -169 and -162 in EII β-glucanase

promoter, TAACAACC, is very similar to the GAMYB binding site with only one base mismatch. Thus, GAMYB may also play a crucial role in regulation of the barley aleurone β-glucanase gene. The sequence between -140 and -135, GAACCGAA, in the cathepsin B-like gene promoter may act as a binding site for a wheat GAMYB homologue.

EXAMPLE 14

Cloning of a cDNA encoding rice *GAmyb*

To isolate cDNA clones encoding rice *GAMyb* (*OsGAMyb*), a cDNA library made from gibberellin-treated embryoless-half grains (Chen *et al.*, 1995) was screened using a partial genomic clone isolated by screening a IR36 rice genomic library (Clontech) with a barley 3′ *GAMyb* probe at low stringency (2 x SSC, 0.1% SDS at 54°C). Positive hybridising clones were identified: Six plaques were picked and isolated for further analysis.

The nucleotide sequence of the rice *GAMyb* (*OsGAMyb*) cDNA clone was determined and is set forth in SEQ ID No: 3. The nucleotide sequence contained a single open reading frame, from residues 396 to 2054 of SEQ ID NO:3, which encodes for a MYB-like polypeptide comprising 553 amino acids residues in length (molecular weight 59 kDa).

Comparison of the amino acid sequences of rice and barley GAMYB polypeptides (i.e. SEQ ID NO:2 compared to SEQ ID NO:4), revealed 88% identity overall(Figure 12). There is very high sequence identity between the DNA binding domains of both MYB polypeptides. Furthermore the R2 and R3 repeats of the amino acid sequences of barley and rice GAMYBs show high sequence identity (99%), indicating that both MYBs are likely to have very similar binding specificities. Sequences downstream of the DNA binding domain up to and including a putative activator domain (amino acid residues 372 to 386 of the *Os*GAMYB amino acid sequence set forth in SEQ ID No: 4) are also highly conserved. Sequences downstream of the activator domain are approximately 75%

conserved in barley and rice GAMYB polypeptides.

Based on the high level of sequence identity at the amino acid level, the rice cDNA is likely to encode the rice homologue of the *HvGAMyb* cDNA clone described herein.

EXAMPLE 15

Analysis of rice *GAMyb* gene expression in response to GA

The effect of GA on rice *GAMyb* (*OsGAMyb*) gene expression was monitored by incubating hydrated endosperm half grains of rice in the presence and absence of GA up to 12h. RNA was extracted and analysed by RNA gel blot analysis using the 3′ *OsGAMyb* PCR product.

As shown in Figure 13a the probe detected only a single band of 3.5 kb in size. The abundance of *OsGAMyb* mRNA levels increased rapidly in response to GA. Within 1h incubation with GA, *OsGAMyb* mRNA levels began to increase and continued to increase up to 12h. In the absence of GA, *OsGAMyb* mRNA levels decreased continuously. Very low levels of *OsGAMyb* mRNA were detected in endosperm of dry grains (data not shown) compared to hydrated grains indicating that mRNA accumulated in the aleurone cells during the 24h hydration period but subsequently declined in the absence of GA.

The accumulation of α-amylase mRNA in response to GA is shown in Figure 3b. The increase in *OsGAMyb* mRNA levels in response to GA, preceded the rise in α-amylase mRNA which is similar to that found for barley *GAMyb* (*HvGAMyb*) gene expression in barley aleurone cells (Example 3).

EXAMPLE 16

The *Os*GAMYB polypeptide is a transcriptional activator of the α-amylase gene promoter

To test whether *Os*GAMYB is a transcriptional activator of α-amylase gene promoters, barley aleurone cells were co-bombarded with the GUS reporter gene fused to a barley low-pI α-amylase promoter (mlo22; Lanahan *et al.*, 1992) and with the *GAMyb* (*OsGAMyb*) effector construct (Figure 14). As shown in Figure 14a, the effector construct Ubi1.OsGAMyb comprised the *OsGAMyb* cDNA operably connected to the constitutive maize ubiquitin1 promoter (Christiansen *et al.*, 1992).

The *Os*GAMyb effector was able to transactivate the low-pI α-amylase promoter both in the presence and absence of GA. As shown in Figure 4b, the effector construct resulted in a 176-fold increase GUS expression in non-GA treated aleurone cells, compared to an effector construct which had no cDNA insert (Ubi1.cass). In construct, the increase in GUS activity observed with the Ubi1.*Os*GAMyb effector construct, in response to overexpression of the *OsGAMyb* cDNA, was only 2-fold higher in GA-treated aleurone cells bombarded with the Ubi.cass effector construct which contains no *GAMyb* sequence. These results indicate that the *OsGAMyb* cDNA encodes a transcriptional activator of the low-pI α-amylase gene promoter.

On the basis of high sequence identity, responsiveness to GA and functional evidence, the *OsGAMyb* gene described herein encodes the orthologue to *HvGAMyb*.

Furthermore, the results presented herein indicate that a MYB or MYB-like protein plays an important role in the hormonal control α-amylase gene expression in rice and barley aleurone cells. Sequence analysis of cereal α-amylase promoters shows that the MYB binding site, TAACAAA box is highly conserved in the promoters of barley, wheat and rice (Huang *et al.* 1990). It is of interest to note that another early gibberellin in response gene has been identified in rice aleurone (Chen *et al.*, 1995). The expression of gene encoding ubiquitin-activating enzyme was shown to respond within 1 h of GA

application.

EXAMPLE 17

Production of antibodies to barley GAMYB

Two synthetic peptides based on the sequence of the barley GAMYB amino acid sequences MYRVKSESDCEMMHC (i.e. amino acids 1-14 of SEQ ID NO:1) and CGAGDTSSHPENLRP (amino acids 481-494 of SEQ ID NO:1) were synthesised at the Biomolecular Resource Facility, John Curtin School of Medical Research, Australian National University, Australia. The peptides were coupled to keyhole limpet haemocyanin with glutaraldehyde and used for immunisation of 3 months-old New Zealand White rabbits. Pre-immune sera were collected prior to the primary immunisation. The rabbits were given boost immunisations at 1, 2, 4, 5 and 10 weeks. Sera were collected and the IgG fractions were purified by Protein G columns as described the manufacturer's instruction (Pharmacia, Upsala).

Pre-immune antibodies and immune antibodies were tested for immunoreactivity to GAMYB fusion protein by Western Blot analysis. The GAMYB fusion protein was prepared as described in Example 7, separated on a 10% (w/v) SDS/polyacrylamide gel and transferred to PVDF membranes. The blots were probed with the purified antibodies followed by horseradish peroxidase linked goat anti-rabbit antibodies (Amersham, England). Blots were developed using a chemiluminescence substrate as described by the manufacturer's instructions (DuPont NEN).

The results presented in Figure 15 show that the immune antibodies raised against both peptide recognise the GAMYB fusion protein.

Western blot analysis of barley grain proteins show that the immune antibodies also bind to native GAMYB protein. Because of the high similarity in amino acid sequence between barley and rice, it is expected that the anti-GAMYB antibodies will cross-react with other cereal GAMYB proteins.

EXAMPLE 18

Antisense GAMyb gene expression in rice plants blocks expression of α-amylase and other hydrolytic genes in aleurone cells in germinating grains

The effector construct, Ubi1.as*Os*GAMyb (Figure 16) was designed to express antisense *GAMyb* mRNA. The construct was made by inserting a fragment containing the 3′ end of *OsGAMyb* cDNA sequence, in the antisense orientation, into the Ubi1.cass construct shown in Figure 14a. Rice plants were transformed with this construct as described by Li *et al.*, (1993).

Transgenic lines were confirmed by Southern blot hybridisation analysis using the 3′ end of *OsGAMyb* as a probe. Figure 17 shows restriction digestion of genomic DNA from transgenic lines probed with 3′ end of the *OsGAMyb* cDNA. The results show that the transgenic lines contain the new construct.

Analysis of grains from the T_1 and subsequent generations show that expression of hydrolytic enzymes in aleurone cells of germinating grains is blocked in transgenic lines expressing antisense *GAMyb* mRNA.

EXAMPLE 19

Barley *GAMyb* gene expression increases early in germination

To test whether *GAMyb* is involved in germination and dormancy, *GAMyb* gene expression was monitored in embryos of germinating barley grains. RNA gel blots containing RNA from various timepoints were probed using a 3′ gene specific *HvGAMyb* cDNA probe. Results were quantified using a Phosphorimager and normalised to rRNA levels (Figure 18).

As shown in Figure 18, *GAMyb* mRNA was detected in embryos of dry mature barley

grains. Following the start of imbibition, *GAMyb* mRNA levels increased 3-fold in the first 12h. The increase in *GAMyb* mRNA levels preceded germination. These results suggest that the barley GAMYB polypeptide is likely to be involved in germination.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: CSIRO, Division of Plant Industry

(ii) TITLE OF INVENTION: Plant Regulatory Proteins

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Davies Collison Cave Patent Attorneys

(B) STREET: 1, Little Collins Street

(C) CITY: Melbourne

(D) STATE: Victoria

(E) COUNTRY: Australia

(F) ZIP: 3000

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:PCT International Application

(B) FILING DATE: 21-JUN-1996

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: AU PN3779/95

(B) FILING DATE: 23-JUN-1995

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:AU PN6470/95

(B) FILING DATE: 9-NOV-1995

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Slattery, John M.

(ix) TELECOMMUNICATION INFORMATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2220 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Hordeum vulgare

(B) STRAIN: Himalaya

(D) DEVELOPMENTAL STAGE: Seed

(F) TISSUE TYPE: Aleurone layer

(vii) IMMEDIATE SOURCE:

(B) CLONE: *HvGAMyb*

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 275..1933

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GCTGCAGCTC AGCTCCGCCC CAGCTCAGCC CCCGGAGCCC GAGCTGATCG ACCCGCCGGC     240

AGCGAGATTC GGCCAGGGCA CGATCGACGG AGAG ATG TAC CGG GTG AAG AGC         292
                                      Met Tyr Arg Val Lys Ser
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GGG CCG CCT CTG AAG AAG GGG CCC TGG ACG TCC GCG GAG GAC GCC ATC        436
Gly Pro Pro Leu Lys Lys Gly Pro Trp Thr Ser Ala Glu Asp Ala Ile
    40                  45                  50

CTG GTG GAC TAC GTG AAG AAG CAC GGC GAG GGG AAC TGG AAC GCG GTG        484
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55                  60                  65                  70

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Trp Ala Asn His Leu Arg Pro Asn Leu Lys Lys Gly Ala Phe Thr Pro
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Glu Glu Glu Arg Leu Ile Ile Gln Leu His Ser Lys Met Gly Asn Lys
        105                 110                 115

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Trp Ala Arg Met Ala Ala His Leu Pro Gly Arg Thr Asp Asn Glu Ile
    120                 125                 130

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Lys Asn Tyr Trp Asn Thr Arg Ile Lys Arg Cys Gln Arg Ala Gly Leu
135                 140                 145                 150

CCA ATA TAT CCT GCT AGT GTA TGC AAT CAA TCT TCA AAT GAA GAT CAG        772
Pro Ile Tyr Pro Ala Ser Val Cys Asn Gln Ser Ser Asn Glu Asp Gln
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185 190 195

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200 205 210

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Ser Ile Ser Ser Leu Leu Gly Gln Ser Phe Ala Ser Lys Asn Cys Gly
215 220 225 230

TTC ATG GAT CAA GTA AAC CAA GCA GGG ATG CTA AAA CAG TCT GAC CCT 1012
Phe Met Asp Gln Val Asn Gln Ala Gly Met Leu Lys Gln Ser Asp Pro
235 240 245

TTA CTC CCT GGA TTG AGC GAC ACC ATC AAT GGC GCG CTC TCC TCG GTC 1060
Leu Leu Pro Gly Leu Ser Asp Thr Ile Asn Gly Ala Leu Ser Ser Val
250 255 260

GAT CAA TTC TCA AAT GAC TCT GAG AAG CTC AAG CAG GCT CTT GGT TTT 1108
Asp Gln Phe Ser Asn Asp Ser Glu Lys Leu Lys Gln Ala Leu Gly Phe
265 270 275

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Asp Tyr Leu His Glu Ala Asn Ser Ser Ser Lys Ile Ile Ala Pro Phe
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295 300 305 310

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315 320 325

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CAA GAT ACC GAA TCT GAT CCG AAT AGC TGG CTC AAG TAT ACC GTG GCT     1300
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CCT GCG ATG CAG CCT ACG GAG TTG GTT GAT CCG TAC CTG CAG TCC CCG     1348
Pro Ala Met Gln Pro Thr Glu Leu Val Asp Pro Tyr Leu Gln Ser Pro
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ACA GCA ACT CCG TCA GTG AAA TCG GAG TGT GCT TCG CCG AGG AAC AGC     1396
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CTC ACT GGA TCC ACC GCT CCT ATG AGT GCT GCG TCG CCT GAT GTT TTT     1636
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CAG CTC TCC AAA ATT TCT CCT GCA CAA AGC CCT TCA CTG GGA TCT GGA     1684
Gln Leu Ser Lys Ile Ser Pro Ala Gln Ser Pro Ser Leu Gly Ser Gly
455                 460                 465                 470

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GCC GAC TCG TCC GTC TTC AAC AAC GCC ATA GCC ATG CTC CTG GGC AAC       1828
Ala Asp Ser Ser Val Phe Asn Asn Ala Ile Ala Met Leu Leu Gly Asn
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Asp Met Asn Thr Glu Cys Lys Pro Val Phe Gly Asp Gly Ile Met Phe
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Glu Phe Lys

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TTTTCCCCAA TAAAGTTGGC GGAGATAAGC CGGTTATCTA TTTTTTGTTT GTTTGTTTCG    2153

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ACTGCTT                                                               2220
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 553 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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            20                  25                  30

Ser Pro His Arg Gly Gly Gly Pro Pro Leu Lys Lys Gly Pro Trp Thr
        35                  40                  45

Ser Ala Glu Asp Ala Ile Leu Val Asp Tyr Val Lys Lys His Gly Glu
    50                  55                  60

Gly Asn Trp Asn Ala Val Gln Lys Asn Thr Gly Leu Phe Arg Cys Gly
65                  70                  75                  80

Lys Ser Cys Arg Leu Arg Trp Ala Asn His Leu Arg Pro Asn Leu Lys
                85                  90                  95

Lys Gly Ala Phe Thr Pro Glu Glu Glu Arg Leu Ile Ile Gln Leu His
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Ser Lys Met Gly Asn Lys Trp Ala Arg Met Ala Ala His Leu Pro Gly
        115                 120                 125

Arg Thr Asp Asn Glu Ile Lys Asn Tyr Trp Asn Thr Arg Ile Lys Arg
    130                 135                 140

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Asn Leu Ser Ser Asp Leu Leu Asn Gly Asn Gly Leu Tyr Leu Pro Asp
            180                 185                 190

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Leu Lys Gln Ser Asp Pro Leu Leu Pro Gly Leu Ser Asp Thr Ile Asn
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420 425 430

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450 455 460

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Gly Ala Gly Asp Thr Ser Ser His Pro Glu Asn Leu Arg Pro Asp Ala
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Phe Phe Ser Gly Asn Thr Ala Asp Ser Ser Val Phe Asn Asn Ala Ile
500 505 510

Ala Met Leu Leu Gly Asn Asp Met Asn Thr Glu Cys Lys Pro Val Phe
515 520 525

Gly Asp Gly Ile Met Phe Asp Thr Ser Val Trp Ser Asn Leu Pro His
530 535 540

Ala Cys Gln Met Ser Glu Glu Phe Lys
545 550

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2352 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Oryza sativa

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 396..2054

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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CCTCTCTTGC TCGTCCGCAA GGTCGCTCCC CCTGCGAGTC CAATCTACTG ACGAGCTGGG     300

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                                         1               5

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            10                  15                  20

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        25                  30                  35

CCG CTG AAG AAG GGG CCA TGG ACG TCG GCG GAG GAC GCC ATC CTG GTG       557
Pro Leu Lys Lys Gly Pro Trp Thr Ser Ala Glu Asp Ala Ile Leu Val
    40                  45                  50

GAC TAC GTG AAG AAG CAC GGC GAG GGG AAC TGG AAC GCG GTG CAG AAG       605
Asp Tyr Val Lys Lys His Gly Glu Gly Asn Trp Asn Ala Val Gln Lys
 55                  60                  65                  70

AAC ACC GGG CTG TTC CGG TGC GGC AAG AGC TGC CGC CTC CGG TGG GCG       653
Asn Thr Gly Leu Phe Arg Cys Gly Lys Ser Cys Arg Leu Arg Trp Ala
                75                  80                  85

AAC CAC CTG AGG CCC AAC CTC AAG AAG GGG GCC TTC ACC GCC GAG GAG       701
Asn His Leu Arg Pro Asn Leu Lys Lys Gly Ala Phe Thr Ala Glu Glu
            90                  95                 100
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GAG AGG CTC ATC ATC CAG CTC CAC TCC AAG ATG GGG AAC AAG TGG GCT        749
Glu Arg Leu Ile Ile Gln Leu His Ser Lys Met Gly Asn Lys Trp Ala
        105                 110                 115

CGG ATG GCC GCT CAT TTG CCA GGG CGC ACT GAT AAT GAA ATA AAG AAT        797
Arg Met Ala Ala His Leu Pro Gly Arg Thr Asp Asn Glu Ile Lys Asn
    120                 125                 130

TAC TGG AAT ACT CGA ATA AAG AGA TGC CAG CGA GCT GGC CTA CCC ATC        845
Tyr Trp Asn Thr Arg Ile Lys Arg Cys Gln Arg Ala Gly Leu Pro Ile
135                 140                 145                 150

TAT CCT ACC AGC GTA TGC AAT CAA TCC TCA AAT GAA GAT CAG CAG TGC        893
Tyr Pro Thr Ser Val Cys Asn Gln Ser Ser Asn Glu Asp Gln Gln Cys
                155                 160                 165

TCC AGT GAT TTT GAC TGT GGC GAG AAT TTG TCA AAC GAT CTT CTG AAT        941
Ser Ser Asp Phe Asp Cys Gly Glu Asn Leu Ser Asn Asp Leu Leu Asn
            170                 175                 180

GCA AAT GGT CTT TAC CTA CCA GAT TTT ACC TGT GAC AAT TTC ATT GCT        989
Ala Asn Gly Leu Tyr Leu Pro Asp Phe Thr Cys Asp Asn Phe Ile Ala
        185                 190                 195

AAT TCA GAG GCT TTA CCT TAT GCA CCA CAT CTT TCA GCC GTT TCT ATA       1037
Asn Ser Glu Ala Leu Pro Tyr Ala Pro His Leu Ser Ala Val Ser Ile
    200                 205                 210

AGC AAT CTC CTT GGC CAG AGC TTT GCA TCA AAA AGC TGT AGC TTC ATG       1085
Ser Asn Leu Leu Gly Gln Ser Phe Ala Ser Lys Ser Cys Ser Phe Met
215                 220                 225                 230

GAT CAG GTA AAC CAG ACA GGG ATG CTA AAA CAG TCT GAT GGT GTG CTT       1133
Asp Gln Val Asn Gln Thr Gly Met Leu Lys Gln Ser Asp Gly Val Leu
                235                 240                 245

CCT GGA TTG AGC GAT ACC ATC AAC GGT GTG ATT TCC TCG GTG GAT CAA       1181
Pro Gly Leu Ser Asp Thr Ile Asn Gly Val Ile Ser Ser Val Asp Gln
            250                 255                 260
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TTC TCA AAT GAC TCT GAG AAG CTC AAG CAG GCT GTG GGT TTT GAC TAT      1229
Phe Ser Asn Asp Ser Glu Lys Leu Lys Gln Ala Val Gly Phe Asp Tyr
        265                 270                 275

CTC CAT GAA GCC AAC TCT ACC AGC AAG ATT ATT GCA CCT TTC GGG GGT      1277
Leu His Glu Ala Asn Ser Thr Ser Lys Ile Ile Ala Pro Phe Gly Gly
    280                 285                 290

GCA CTT AAT GGC AGC CAT GCC TTT TTA AAT GGC AAT TTC TCT GCT TCT      1325
Ala Leu Asn Gly Ser His Ala Phe Leu Asn Gly Asn Phe Ser Ala Ser
295                 300                 305                 310

AGG CCC ACA AGT GGT CCT TTG AAG ATG GAG CTC CCT TCA CTC CAA GAT      1373
Arg Pro Thr Ser Gly Pro Leu Lys Met Glu Leu Pro Ser Leu Gln Asp
                315                 320                 325

ACT GAA TCT GAT CCA AAC AGC TGG CTC AAG TAC ACT GTA GCT CCT GCG      1421
Thr Glu Ser Asp Pro Asn Ser Trp Leu Lys Tyr Thr Val Ala Pro Ala
            330                 335                 340

TTG CAG CCT ACT GAG TTA GTT GAT CCC TAC CTG CAG TCT CCA GCA GCA      1469
Leu Gln Pro Thr Glu Leu Val Asp Pro Tyr Leu Gln Ser Pro Ala Ala
        345                 350                 355

ACC CCT TCA GTG AAA TCA GAG TGC GCG TCG CCA AGG AAT AGT GGC CTT      1517
Thr Pro Ser Val Lys Ser Glu Cys Ala Ser Pro Arg Asn Ser Gly Leu
    360                 365                 370

TTG GAA GAG TTG ATT CAT GAA GCT CAG ACC CTA AGA TCC GGG AAG AAC      1565
Leu Glu Glu Leu Ile His Glu Ala Gln Thr Leu Arg Ser Gly Lys Asn
375                 380                 385                 390

CAA CAG ACA TCT GTG ATA AGT TCT AGT TCT TCT GTC GGT ACG CCA TGT      1613
Gln Gln Thr Ser Val Ile Ser Ser Ser Ser Ser Val Gly Thr Pro Cys
                395                 400                 405

AAT ACT ACG GTT CTT AGC CCA GAG TTT GAT ATG TGT CAG GAA TAC TGG      1661
Asn Thr Thr Val Leu Ser Pro Glu Phe Asp Met Cys Gln Glu Tyr Trp
            410                 415                 420
```

```
GAA GAA CAA CAT CCT GGT CCA TTC CTC AAT GAC TGT GCT CCT TTC AGT      1709
Glu Glu Gln His Pro Gly Pro Phe Leu Asn Asp Cys Ala Pro Phe Ser
        425                 430                 435

GGC AAT TCA TTC ACT GAA TCC ACC CCT CCT GTT AGC GCT GCA TCG CCT      1757
Gly Asn Ser Phe Thr Glu Ser Thr Pro Pro Val Ser Ala Ala Ser Pro
    440                 445                 450

GAC ATC TTT CAG CTC TCC AAA GTT TCC CCA GCA CAA AGC ACT TCA ATG      1805
Asp Ile Phe Gln Leu Ser Lys Val Ser Pro Ala Gln Ser Thr Ser Met
455                 460                 465                 470

GGA TCT GGA GAG CAA GTA ATG GGG CCT AAA TAT GAA CCT GGG GAC ACT      1853
Gly Ser Gly Glu Gln Val Met Gly Pro Lys Tyr Glu Pro Gly Asp Thr
                475                 480                 485

TCA CCT CAT CCT GAA AAC TTC AGG CCA GAT GCA TTG TTT TCT GGG AAT      1901
Ser Pro His Pro Glu Asn Phe Arg Pro Asp Ala Leu Phe Ser Gly Asn
            490                 495                 500

ACA GCT GAT CCA TCA GTT TTC AAC AAT GCC ATA GCA ATG CTT CTG GGC      1949
Thr Ala Asp Pro Ser Val Phe Asn Asn Ala Ile Ala Met Leu Leu Gly
        505                 510                 515

AAT GAC TTG AGT ATC GAT TGC AGA CCT GTT CTT GGC GAC GGT ATC ATG      1997
Asn Asp Leu Ser Ile Asp Cys Arg Pro Val Leu Gly Asp Gly Ile Met
    520                 525                 530

TTC AAT TCT TCC TCG TGG AGC AAC ATG CCA CAC GCC TGT GAA ATG TCA      2045
Phe Asn Ser Ser Ser Trp Ser Asn Met Pro His Ala Cys Glu Met Ser
535                 540                 545                 550

GAA TTC AAA TGAATTCTGT ATTTGGTATT TGCCAGACGC TAAAGCAGAT              2094
Glu Phe Lys

TCTTGGTATC GTTCTTGCTC ATTGTTTGGA TCATAACTTG AGGAAGATCT CGTCGGTTGT    2154

AATTCTGCAT TGCTGACAGA GTCCTTGATG GCATGCAGGC ACTATCAGAT GTTCTCTTAT    2214

GCGACTAGCC CTTTTGTCCA ATAAAGTAGT GCATGGAGAT AAGCCGGTTA ATTATTGTTT    2274
```

TCTTTTTTGT ATTAGAGAAC CCTTTTTGTC ATCTCTATTG CCGGAATTCT GTTGGAAATT 2334

CGTCATGTTT GTTTGAAC 2352

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 553 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Tyr Arg Val Lys Ser Glu Ser Asp Cys Glu Met Ile His Gln Glu
  1               5                  10                  15

Gln Met Asp Ser Pro Val Ala Asp Asp Gly Ser Ser Gly Gly Ser Pro
             20                  25                  30

His Arg Gly Gly Gly Pro Pro Leu Lys Lys Gly Pro Trp Thr Ser Ala
         35                  40                  45

Glu Asp Ala Ile Leu Val Asp Tyr Val Lys Lys His Gly Glu Gly Asn
     50                  55                  60

Trp Asn Ala Val Gln Lys Asn Thr Gly Leu Phe Arg Cys Gly Lys Ser
 65                  70                  75                  80

Cys Arg Leu Arg Trp Ala Asn His Leu Arg Pro Asn Leu Lys Lys Gly
                 85                  90                  95

Ala Phe Thr Ala Glu Glu Glu Arg Leu Ile Ile Gln Leu His Ser Lys
            100                 105                 110

Met Gly Asn Lys Trp Ala Arg Met Ala Ala His Leu Pro Gly Arg Thr
        115                 120                 125
```

Asp Asn Glu Ile Lys Asn Tyr Trp Asn Thr Arg Ile Lys Arg Cys Gln
130 135 140

Arg Ala Gly Leu Pro Ile Tyr Pro Thr Ser Val Cys Asn Gln Ser Ser
145 150 155 160

Asn Glu Asp Gln Gln Cys Ser Ser Asp Phe Asp Cys Gly Glu Asn Leu
165 170 175

Ser Asn Asp Leu Leu Asn Ala Asn Gly Leu Tyr Leu Pro Asp Phe Thr
180 185 190

Cys Asp Asn Phe Ile Ala Asn Ser Glu Ala Leu Pro Tyr Ala Pro His
195 200 205

Leu Ser Ala Val Ser Ile Ser Asn Leu Leu Gly Gln Ser Phe Ala Ser
210 215 220

Lys Ser Cys Ser Phe Met Asp Gln Val Asn Gln Thr Gly Met Leu Lys
225 230 235 240

Gln Ser Asp Gly Val Leu Pro Gly Leu Ser Asp Thr Ile Asn Gly Val
245 250 255

Ile Ser Ser Val Asp Gln Phe Ser Asn Asp Ser Glu Lys Leu Lys Gln
260 265 270

Ala Val Gly Phe Asp Tyr Leu His Glu Ala Asn Ser Thr Ser Lys Ile
275 280 285

Ile Ala Pro Phe Gly Gly Ala Leu Asn Gly Ser His Ala Phe Leu Asn
290 295 300

Gly Asn Phe Ser Ala Ser Arg Pro Thr Ser Gly Pro Leu Lys Met Glu
305 310 315 320

Leu Pro Ser Leu Gln Asp Thr Glu Ser Asp Pro Asn Ser Trp Leu Lys
325 330 335

Tyr Thr Val Ala Pro Ala Leu Gln Pro Thr Glu Leu Val Asp Pro Tyr
340 345 350

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Leu Gln Ser Pro Ala Ala Thr Pro Ser Val Lys Ser Glu Cys Ala Ser
        355                     360                     365

Pro Arg Asn Ser Gly Leu Leu Glu Glu Leu Ile His Glu Ala Gln Thr
    370                     375                     380

Leu Arg Ser Gly Lys Asn Gln Gln Thr Ser Val Ile Ser Ser Ser Ser
385                     390                     395                     400

Ser Val Gly Thr Pro Cys Asn Thr Thr Val Leu Ser Pro Glu Phe Asp
                405                     410                     415

Met Cys Gln Glu Tyr Trp Glu Glu Gln His Pro Gly Pro Phe Leu Asn
            420                     425                     430

Asp Cys Ala Pro Phe Ser Gly Asn Ser Phe Thr Glu Ser Thr Pro Pro
        435                     440                     445

Val Ser Ala Ala Ser Pro Asp Ile Phe Gln Leu Ser Lys Val Ser Pro
    450                     455                     460

Ala Gln Ser Thr Ser Met Gly Ser Gly Glu Gln Val Met Gly Pro Lys
465                     470                     475                     480

Tyr Glu Pro Gly Asp Thr Ser Pro His Pro Glu Asn Phe Arg Pro Asp
                485                     490                     495

Ala Leu Phe Ser Gly Asn Thr Ala Asp Pro Ser Val Phe Asn Asn Ala
            500                     505                     510

Ile Ala Met Leu Leu Gly Asn Asp Leu Ser Ile Asp Cys Arg Pro Val
        515                     520                     525

Leu Gly Asp Gly Ile Met Phe Asn Ser Ser Ser Trp Ser Asn Met Pro
    530                     535                     540

His Ala Cys Glu Met Ser Glu Phe Lys
545                     550
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CLAIMS:

1. An isolated nucleic acid molecule comprising a sequence which encodes or is complementary to a sequence which encodes a gibberellin-regulated MYB polypeptide or a homologue, analogue or derivative thereof.

2. The isolated nucleic acid molecule according to claim 1, wherein said gibberellin-regulated MYB polypeptide is at least expressed in the seeds of plants.

3. The isolated nucleic acid molecule according to claim 2 wherein said MYB polypeptide regulates the expression of gibberellin-regulated genes encoding hydrolytic enzymes involved in the malting process.

4. The isolated nucleic acid molecule according to claim 3 wherein the hydrolytic enzymes are selected from the list comprising the high pI α-amylase, low pI α-amylase, EII-(1-3,1-4)-β-glucanase, cathepsin β-like protease, α-glucosidase, xylanase and arabinofuranosidase, amongst others.

5. The isolated nucleic acid molecule according to claim 4 wherein the hydrolytic enzyme is α-amylase.

6. The isolated nucleic acid molecule according to any one of claims 1 to 5 comprising deoxyribonucleotides.

7. The isolated nucleic acid molecule according to any one of claims 1 to 6 when derived from a monocotyledonous plant species selected from the list comprising rice, barley, wheat, maize, rye and sorghum, amongst others.

8. The isolated nucleic acid molecule according to claim 7 when derived from a rice plant.

9. The isolated nucleic acid m lecule according to claim 7 when derived from a barley plant.

10. An isolated nucleic acid molecule comprising a sequence of nucleotides corresponding or complementary to the nucleotide sequence set forth in SEQ ID No:1 or a homologue, analogue or derivative thereof or having at least 40% nucleotide sequence similarity thereto.

11. An isolated nucleic acid molecule comprising a sequence of nucleotides corresponding or complementary to the nucleotide sequence set forth in SEQ ID No:3 or a homologue, analogue or derivative thereof or having at least 40% nucleotide sequence similarity thereto.

12. The isolated nucleic acid molecule according to claim 10 or 11 wherein the percentage similarity is at least 55%.

13. The isolated nucleic acid molecule according to claim 12 wherein the percentage similarity is at least 75-80%.

14. The isolated nucleic acid molecule according to claim 13 wherein the percentage similarity is at least 85-95%.

15. The isolated nucleic acid molecule according to any one of claims 10 to 14 further characterised as comprising a sequence of nucleotides which encodes or is complementary to a nucleotide sequence which encodes a plant gibberellin-regulated MYB polypeptide as hereinbefore defined.

16. An isolated nucleic acid molecule which is capable of hybridising under at least low stringency hybridisation conditions as hereinbefore defined to the nucleotide sequence set forth in SEQ ID No:1 or SEQ ID No:3 or a complementary nucleotide sequence, homologue, analogue or derivative thereof.

17. The isolated nucleic acid molecule according to claim 16 further comprising a nucleotide sequence which encodes a plant GAMYB polypeptide as hereinbefore defined.

18. The isolated nucleic acid molecule according to claim 17 wherein the plant is a barley, rice, wheat, maize, rye or sorghum plant.

19. An isolated or recombinant polypeptide comprising an amino acid sequence having the transcriptional activation function of a gibberellin-regulated MYB as hereinbefore defined or a functional homologue, analogue or derivative thereof.

20. The isolated or recombinant polypeptide according to claim 19 wherein said polypeptide is the product of a barley or rice *GAMyb* genetic sequence.

21. An isolated or recombinant polypeptide comprising an amino acid sequence which is at least 40% identical to the amino acid sequence set forth in SEQ ID No: 2 or SEQ ID No: 4 or a homologue, analogue or derivative thereof.

22. An isolated or recombinant gibberellin-regulated MYB polypeptide comprising conserved R2 and R3 domains as hereinbefore defined which are substantially the same or at least 85% identical to the R2 and R3 domains of a GAMYB polypeptide derivable from a monocotyledonous plant.

23. The isolated or recombinant polypeptide according to claim 22 wherein the monocotyledonous plant is a barley, maize, wheat, rice, rye or sorghum plant.

24. The isolated or recombinant polypeptide according to claim 23 wherein the monocotyledonous plant is rice or barley.

25. The isolated or recombinant polypeptide according to any one of claims 22 to 24, further comprising a sequence of amino acids which:

(i)is at least 85% identical to residues 42 to 145 of SEQ ID No: 2 or residues 93

to 143 of SEQ ID No: 4 or a homologue, analogue or derivative thereof; and (ii) is at least 40% identical to amino acid residues contained in SEQ ID No: 2 or SEQ ID No: 4 or a homologue, analogue or derivative thereof, other than amino acid residues defined by paragraph (i).

26. The isolated or recombinant polypeptide according to claim 25 wherein the percentage identity defined by paragraph (ii) is at least 75%.

27. A synthetic peptide comprising at least 10 contiguous amino acid residues derived from SEQ ID No: 2 or SEQ ID No: 4 or a homologue, analogue or derivative thereof which is at least 40% similar thereto.

28. The synthetic peptide according to claim 27 further comprising the sequence MYRVKSESDCEMMHC or a sequence which is at least 40% identical thereto.

29. The synthetic peptide according to claim 27 further comprising the sequence CGAGDTSSHPENLRP or a sequence which is at least 40% identical thereto.

30. An antibody which is capable of binding to a polypeptide according to any one of claims 19 to 26.

31. An antibody which is capable of binding of a synthetic peptide according to any one of claims 27 to 29.

32. A genetic construct comprising the coding region of a gibberellin-regulated *Myb* genetic sequence placed operably in connection with a promoter, in the sense orientation such that a MYB polypeptide or a homologue, analogue or derivative thereof is capable of being expressed under the control of said promoter.

33. The genetic construct according to claim 32 wherein the *Myb* genetic sequence is the barley *GAMyb* sequence set forth in SEQ ID No: 1 or the rice *GAMyb* sequence set

forth in SEQ ID No: 3.

34. The genetic construct according to claim 32 or 33 wherein the promoter is a viral, fungal, bacterial, animal or plant gene promoter.

35. The genetic construct according to claim 34 wherein the promoter is further capable of regulating the expression of a MYB in a plant cell.

36. The genetic construct according to claim 35 wherein the plant cell is a monocotyledonous plant cell.

37. The genetic construct according to claim 36 wherein the monocotyledonous plant is selected from the list comprising wheat, maize, rice, rye, sorghum or barley, amongst others.

38. The genetic construct according to any one of claim 34 to 37 wherein the promoter is selected from the list comprising *HvGAMyb*, *OsGAMyb*, CaMV 35S, NOS, OCS, rice *Actin*1, *Ubiquitin*1, α-amylase, EII -(1-3, 1-4)-β-glucanase or *tac*.

39. An antisense genetic construct comprising at least 10-20 contiguous nucleotides of SEQ ID No: 1 or SEQ ID No: 3 or a homologue, analogue or derivative thereof in the antisense orientation in operable connection with a promoter sequence.

40. The genetic construct according to claim 39 comprising at least 50-100 contiguous nucleotides of SEQ ID No: 1 or SEQ ID No: 3 or a homologue, analogue or derivative thereof.

41. The genetic construct according to claim 40 comprising at least 1000-1500 contiguous nucleotides of SEQ ID No: 1 or SEQ ID No: 3 or a homologue, analogue or derivative thereof.

42. The genetic construct according to claim 41, wherein the region of SEQ ID No: 3 at least comprises nucleotide residues 1003 to 2113.

43. The genetic construct according to any one of claims 39 to 42 wherein the promoter is a viral, bacterial, animal or plant gene promoter.

44. The genetic construct according to claim 43 wherein the promoter is capable of regulating the expression of the antisense molecule comprising nucleotides of SEQ ID No: 1 or SEQ ID No: 3 or a homologue, analogue or derivative thereof in a monocotyledonous plant cell.

45. The genetic construct according to claim 44 wherein the monocotyledonous plant cell is selected from the list comprising wheat, maize, rice, rye, sorghum or barley, amongst others.

46. The genetic construct according to any one of claims 43 to 45 wherein the promoter is selected from the list comprising *HvGAMyb, OsGAMyb,* CaMV 35S, NOS, OCS, rice *Actin*1, *Ubiquitin*1, α-amylase, EII-(1-3,1-4)-β-glucanase or *tac*.

47. The genetic construct according to any one of claims 32 to 38 when introduced into a plant cell to increase the expression of GAMYB in said cell or a whole plant comprising said cell.

48. The genetic construct according to any one of claims 39 to 46 when introduced into a plant cell to decrease, delay or otherwise reduce the expression of a GAMYB in said cell or a whole plant comprising said cell.

49. The genetic construct according to claim 48 wherein the expression is reduced by at least 20% compared to the expression of a GAMYB in an isogenic non-transformed plant or other plant.

50. The genetic construct according to claim 49 wherein expression is reduced by at least 50%.

51. The genetic construct according to claim 50 wherein expression is reduced by at least 75%.

52. The genetic construct according to claim 51 wherein expression is reduced by at least 90%.

53. The genetic construct according to any one of claims 47 to 52 wherein increased or reduced expression of a GAMYB polypeptide leads to altered expression of a gene which is regulated by a GAMYB polypeptide.

54. The genetic construct according to claim 53 wherein the gene regulated by a GAMYB is selected from the list comprising genes involved in stem elongation, flowering, leaf development, fruit set and growth, sex determination, germination or malting.

55. The genetic construct according to claim 54 wherein the gene encodes a hydrolytic malting enzyme selected from the list comprising α-amylase, β-glucanase including EII-(1-3,1-4)-β-glucanase, α-glucosidase, xylanase, cathepsin β-like protease or arabinofuranosidase, amongst others.

56. A ribozyme molecule which is capable of cleaving mRNA encoding a GAMYB polypeptide, said ribozyme comprising:

(i) a hybridising region consisting of at least two domains each of which comprises a sequence complementary to at least 5 contiguous nucleotides SEQ ID No: 1 or SEQ ID No: 3 or a homologue, analogue or derivative thereof, wherein said hybridising region is capable of binding to said mRNA; and

(ii) a sequence of nucleotides placed between each domain of the hybridising region, wherein said sequence possesses endoribonuclease activity.

57. A genetic construct capable of expressing the ribozyme of claim 56 under the control of a promoter.

58. The genetic construct of claim 57 wherein the promoter is capable of controlling expression in a plant cell.

59. The genetic construct of claim 58 wherein the plant cell is a monocotyledonous plant cell.

60. The genetic construct of claim 59 wherein the monocotyledonous plant cell is selected from the list comprising barley, wheat, maize, rice, rye or sorghum, amongst others.

61. The genetic construct according to any one of claims 57 to 60 wherein the promoter is selected from the list comprising *HvGAMyb, OsGAMyb,* CaMV 35S, NOS, OCS, rice *Actin*1, *Ubiquitin*1, α-amylase, EII -(1-3, 1-4)-β-glucanase or *tac*.

62. A bacterial cell transformed with the isolated nucleic acid molecule according to any one of claims 1 to 18 or the genetic contruct according to any one of claims 32 to 55 or 57 to 61.

63. A plant cell transformed with the isolated nucleic acid molecule according to any one of claims 1 to 18 or the genetic contruct according to any one of claims 32 to 55 or 57 to 61.

64. The cell according to claim 63 wherein the plant is a monocotyledonous plant species.

65. The cell according to claim 64 wherein the plant is selected from the list comprising barley, rice, maize, wheat, sorghum or rye, amongst others.

66. A transgenic plant regenerated from the cell according to claim 65.

67. A progeny plant of the transformed plant according to claim 66 wherein said progeny plant carries the nucleic acid molecule or genetic construct which present in said transformed plant or a homologue, analogue or derivative thereof.

68. The transgenic plant of claim 64 or the progeny plant of claim 67 wherein said plant exhibits alterd expression of a gene which is involved in one or more processes controlled by a gibberellin-regulated MYB polypeptide and selected from the list comprising stem elongation, flowering, leaf development, fruit set and growth, sex determination, germination or malting characteristics, amongst others.

69 The transgenic plant according to claim 68 wherein the malting characteristic is selected from the list comprising dormancy, germination, post-kilning levels of hydrolytic enzymes, mash filtration properties, precipitate formation and alcohol content, amongst others.

70. The transgenic plant according to claim 69 wherein the hydrolytic enzyme is selected from the list comprising high pI α-amylase, low pI α-amylase, EII-(1-3,1-4)-β-glucanase, cathepsin β-like protease, α-glucosidase, xylanase and arabinofuranosidase, amongst others.

71. A method of modifying the expression of a GAMYB-regulated gene or GAMYB-regulated polypeptide in a plant comprising the steps of:

(i) transforming a plant cell with the genetic construct according to any one of claims 31 to 53 or 55 to 59; and

(ii) regenerating said cell into a whole plant.

72. The method according to claim 71 wherein the GAMYB-regulated gene or GAMYB-regulated polypeptide is involved in a plant developmental process selected from the list comprising stem elongation, flowering, leaf development, fruit set and

growth, sex determination, germination, amongst others, or determines a malting characteristic of a plant.

73. The method according to claim 72 wherein the malting characteristic is selected from the list comprising dormancy, germination, post-kilning levels of hydrolytic enzymes, mash filtration properties, precipitate formation and alcohol content, amongst others.

74. The method according to claim 73 wherein the hydrolytic enzyme is selected from the list comprising high pI α-amylase, low pI α-amylase, EII-(1-3,1-4)-β-glucanase, cathepsin β-like protease, α-glucosidase, xylanase and arabinofuranosidase, amongst others.

75. A method of modifying the malting characteristics of a monocotyledonous plant, plant seed or other plant organ comprising the steps of:

(i) transforming a plant cell with the genetic construct according to any one of claims 31 to 53 or 55 to 59; and

(ii) regenerating said cell into a whole plant.

76. The method according to claim 75 wherein the malting characteristics are selected from the list comprising dormancy, germination, post-kilning levels of hydrolytic enzymes, mash filtration properties, precipitate formation and alcohol content, amongst others.

77. The method according to claim 76, wherein the modified malting characteristic leads to more rapid filtration of the mash.

78. The method according to claim 76, wherein the modified malting characteristic leads to more uniform germination of seed during malting.

79. The method according to claim 76, wherein the modified malting characteristic

leads to reduced cloudy preceipitate formation in the product of the malting process.

80. The method according to claim 76, wherein the modified malting characteristic leads to higher or lower alcohol content in the product of the malting process.

81. The method according to claim 76, wherein the modified malting characteristic leads to high post-kilnin levels of hydrolytic enzmyes required during malting.

82. The method according to claim 81 wherein the hydrolytic enzyme is selected from the list comprising high pI α-amylase, low pI α-amylase, EII-(1-3,1-4)-β-glucanase, cathepsin β-like protease, α-glucosidase, xylanase and arabinofuranosidase, amongst others.

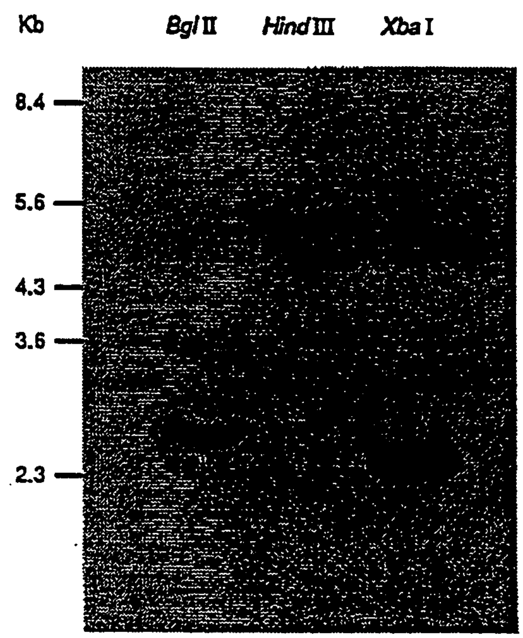


Figure 1

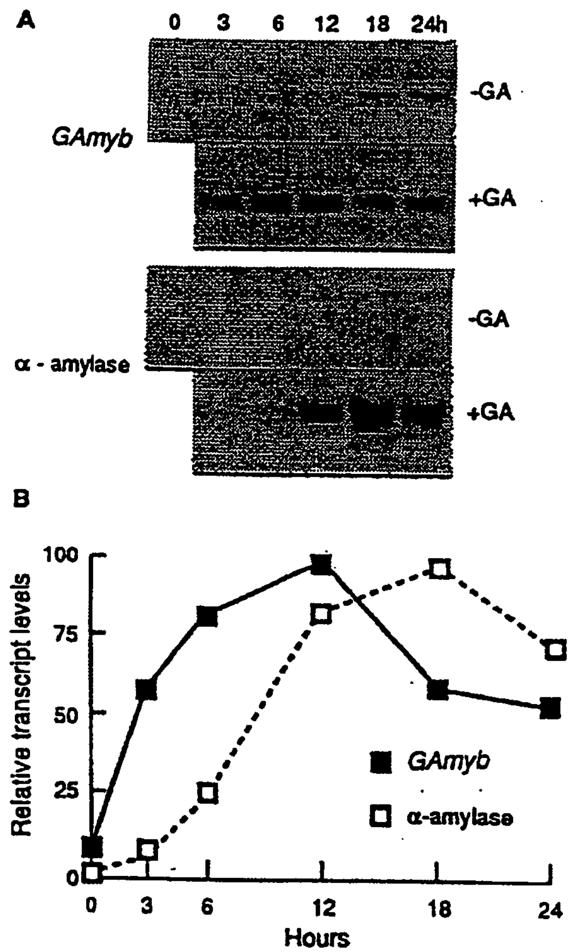

A
0
3
6
12
18
24h
GAmyb
-GA
+GA
α - amylase
-GA
+GA
B
Relative transcript levels
100
75
50
25
0
0
3
6
12
18
24
Hours
GAmyb
α-amylase

Figure 2

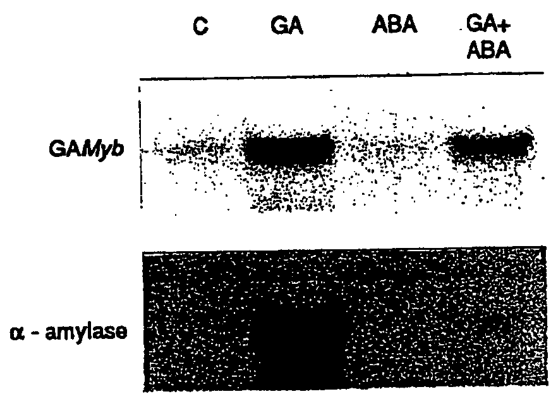


Figure 3

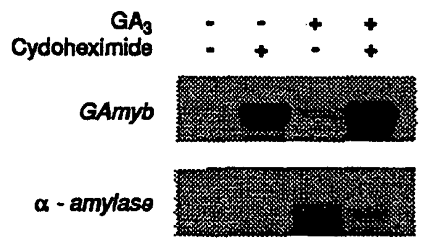


Figure 4

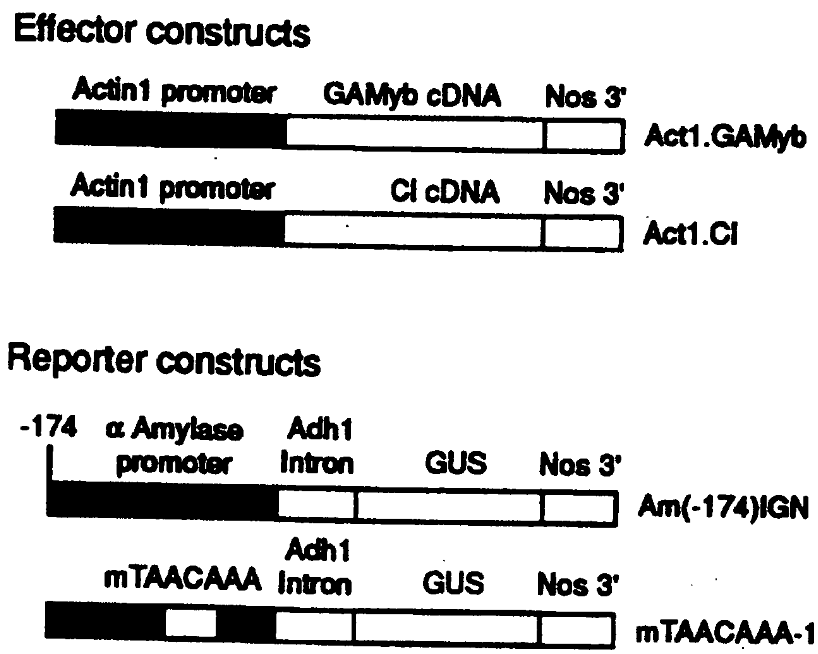

Effector constructs
Actin1 promoter
GAMyb cDNA
Nos 3'
Act1.GAMyb
Actin1 promoter
CI cDNA
Nos 3'
Act1.CI
Reporter constructs
-174
α Amylase promoter
Adh1 Intron
GUS
Nos 3'
Am(-174)IGN
mTAACAAA
Adh1 Intron
GUS
Nos 3'
mTAACAAA-1

Figure 5

A

```
GGCCGATAACAAACTCCGGCCG  w
......CTCGAG..........  m1
.......G..............  m2
...........G..........  m3
............G.........  m4
.............A........  m5
```

B

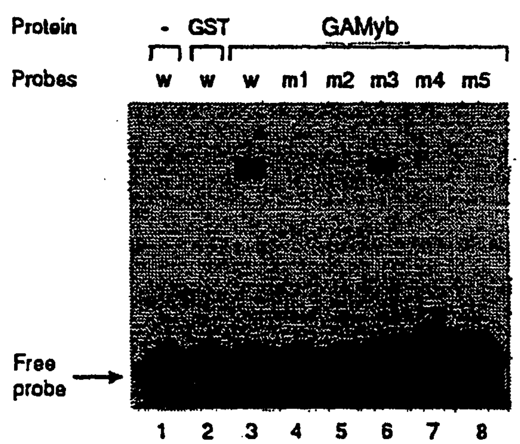


Figure 6

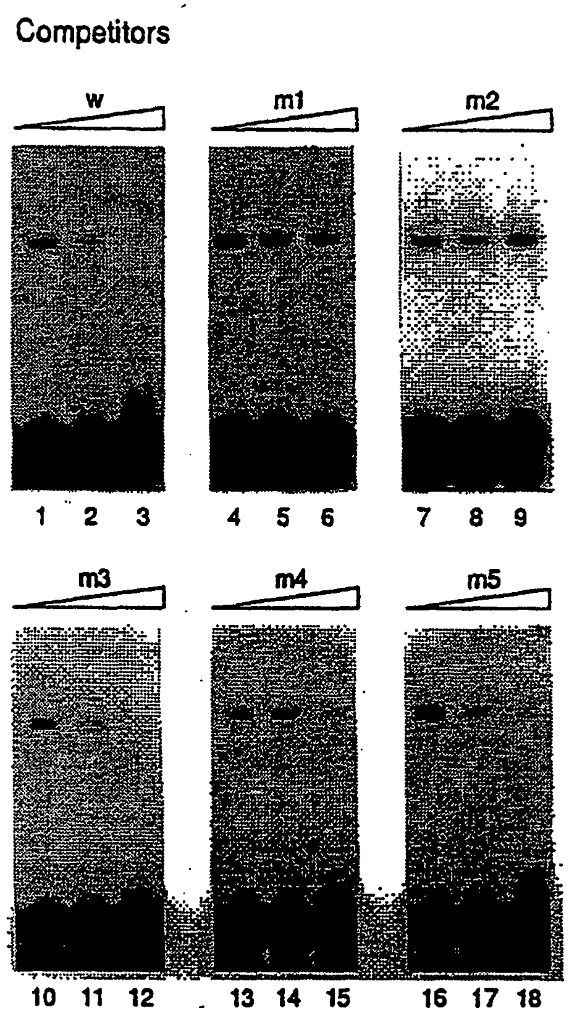


Figure 7

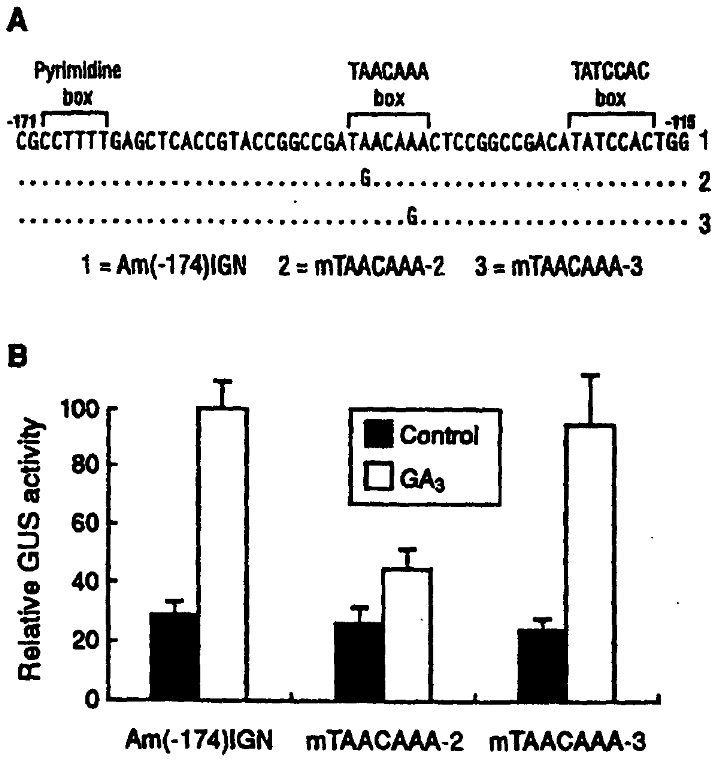

A
Pyrimidine box
TAACAAA box
TATCCAC box
-171
-115
CGCCTTTTGAGCTCACCGTACCGGCCGATAACAAACTCCGGCCGACATATCCACTGG 1
..........................G......................... 2
..............................G..................... 3
1 = Am(-174)IGN 2 = mTAACAAA-2 3 = mTAACAAA-3
B
Relative GUS activity
100
80
60
40
20
0
Control
GA3
Am(-174)IGN
mTAACAAA-2
mTAACAAA-3

Figure 8

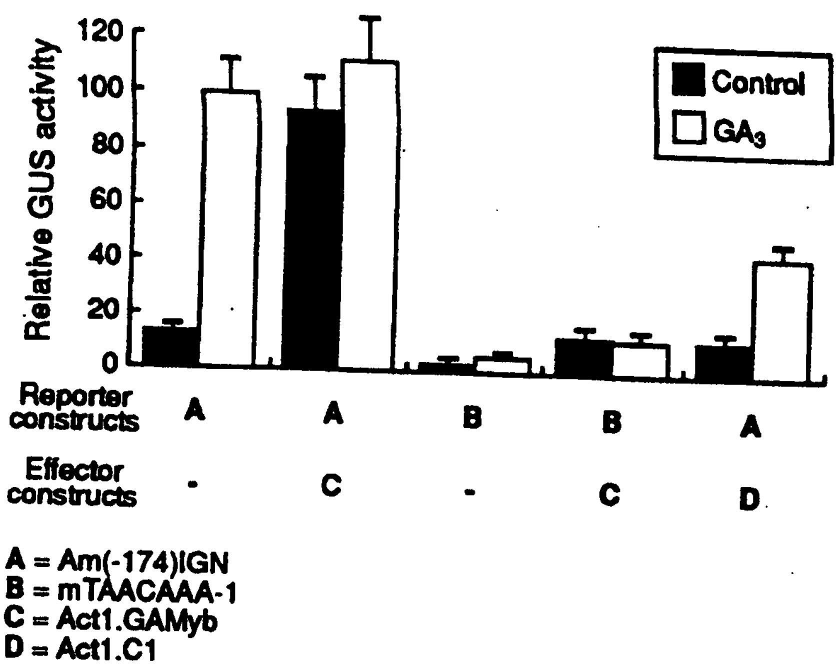

Relative GUS activity
120
100
80
60
40
20
0
Control
GA3
Reporter constructs
A
A
B
B
A
Effector constructs
-
C
-
C
D
A = Am(-174)IGN
B = mTAACAAA-1
C = Act1.GAMyb
D = Act1.C1

Figure 9

Constructs	Without Hormone	10^{-6}M GA_3
EII.IGN + p113 Act1.cas	24.2 ± 4.9	100.0 ± 17.7
EII.IGN + Act1.*GAmyb*	554.5 ± 107.6	803.3 ± 186.5
mlo22 + p113Act1.cas	4.7 ± 1.3	100.0 ± 23.4
mlo 22 + Act1.*GAmyb*	153.3 ± 27.1	229.0 ± 35.3
CBG1 + p113 Act.cas	7.1 ± 3.8	100.0 ± 17.2
CBG1 + Act1.*GAmyb*	118.1 ± 29.0	232.1 ± 65.8

Constucts	Without Hormone	10^{-6}M ABA
Dhn7(-935).IGN + p113 Act1.cas	5.6 ± 1.4	100.0 ± 25.0
Dhn7(-935).IGN + Act1.*GAmyb*	28.4 ± 7.0	131.6 ± 19.8

Figure 10

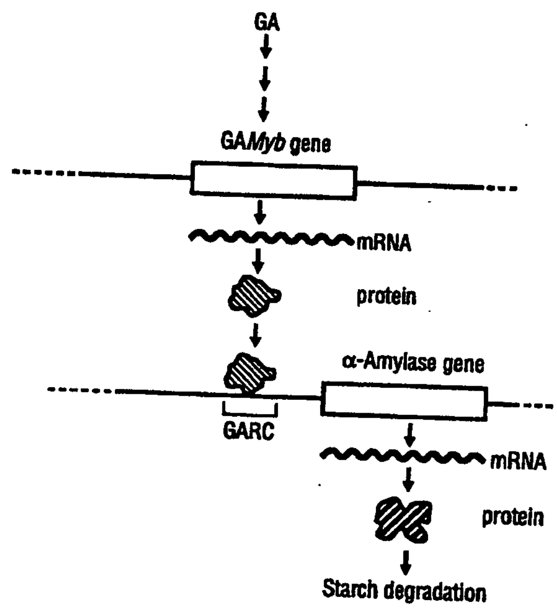

GA
GAMyb gene
mRNA
protein
α-Amylase gene
GARC
mRNA
protein
Starch degradation

Figure 11

```
MYRVKSESDCEMIHQE.QMDSPVADDGSS.GGSPHRGGGPPLKKGPWTSA 48
||||||||||||:||| ||||||:||||| ||||||||||||||||||||
MYRVKSESDCEMMHQEDQMDSPVGDDGSSGGGSPHRGGGPPLKKGPWTSA 50

EDAILVDYVKKHGEGNWNAVQKNTGLFRCGKSCRLRWANHLRPNLKKGAF 98
||||||||||||||||||||||||||||||||||||||||||||||||||
EDAILVDYVKKHGEGNWNAVQKNTGLFRCGKSCRLRWANHLRPNLKKGAF 100

TAEEERLIIQLHSKMGNKWARMAAHLPGRTDNEIKNYWNTRIKRQQRAGL 148
|:||||||||||||||||||||||||||||||||||||||||||||||||
TPEEERLIIQLHSKMGNKWARMAAHLPGRTDNEIKNYWNTRIKRQQRAGL 150

PIYPTSVCNQSSNEDQQCSSDFDCGENLSNDLLNANGLYLPDFTCDNFIA 198
||||.||||||||||||.||||:||||||.||||:|||||||||||||||
PIYPASVCNQSSNEDQQGSSDFNCGENLSSDLLNGNGLYLPDFTCDNFIA 200

NSEALPYAPHLSAVSISNLLGQSFASKSCSFMDQVNQTGMLKQSDGVLPG 248
|||||.|||:|||||||.|||||||||.|:|||||||.|||||||.:|||
NSEALSYAPQLSAVSISSLLGQSFASKNCGFMDQVNQAGMLKQSDPLLPG 250

LSDTINGVISSVDQFSNDSEKLKQAVGFDYLHEANSTSKIIAPFGGALNG 298
|||||||.:||||||||||||||||:||||||||||.|||||||||||.|
LSDTINGALSSVDQFSNDSEKLKQALGFDYLHEANSSSKIIAPFGGALTG 300

SHAFLNGNFSASRPTSGPLKMELPSLQDTESDPNSWLKYTVAPALQPTEL 348
|||||||.||.||...||||||||||||||||||||||||||||:|||||
SHAFLNGTFSTSRTINGPLKMELPSLQDTESDPNSWLKYTVAPAMQPTEL 350

VDPYLQSPAATPSVKSECASPRNSGLLEELIHEAQTLRSGKNQQTSVISS 398
||||||||.|||||||||||||||||||||:||||.|||||||| || ||
VDPYLQSPTATPSVKSECASPRNSGLLEELLHEAQGLRSGKNQQLSVRSS 400

SSSVGTPCNTTVLSPEFDMCQEYWEEQHPGPFLNDCAPFSGNSFTESTPP 448
||||:|||:|||:|||||:|||||||.     ||::|||||||:|:||:|
SSSVSTPCDTTVVSPEFDLCQEYWEER.....LNEYAPFSGNSLTGSTAP 445

VSAASPDIFQLSKVSPAQSTSMGSGEQVMGPKYEP..GDTSPHPENFRPD 496
:||||||:|||||:|||||.|:|||||.|:| |||  ||||.||||:|||
MSAASPDVFQLSKISPAQSPSLGSGEQAMEPAYEPGAGDTSSHPENLRPD 495

ALFSGNTADPSVFNNAIAMLLGNDLSIDCRPVLGDGIMFNSSSWSNMPHA 546
|:|||||||.||||||||||||||:..:|:||:||||||:.| |||:|||
AFFSGNTADSSVFNNAIAMLLGNDMNTECKPVFGDGIMFDTSVWSNLPHA 545

CEMS.EFK 553
|:|| |||
CQMSEEFK 553
```

Figure 12

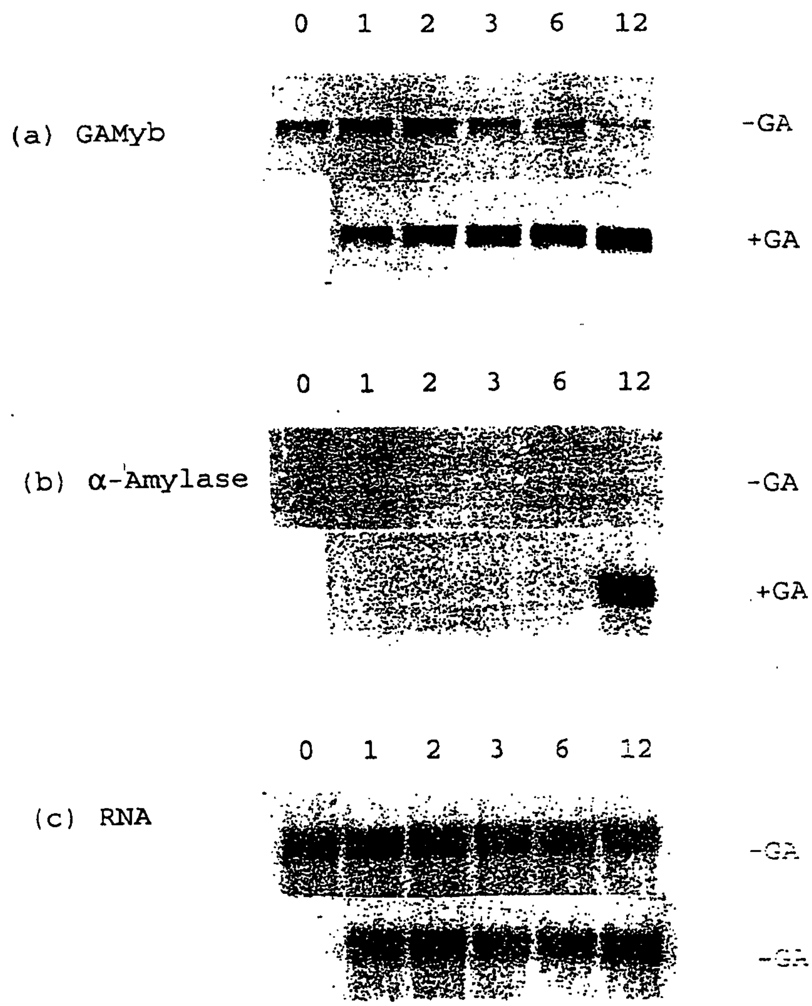
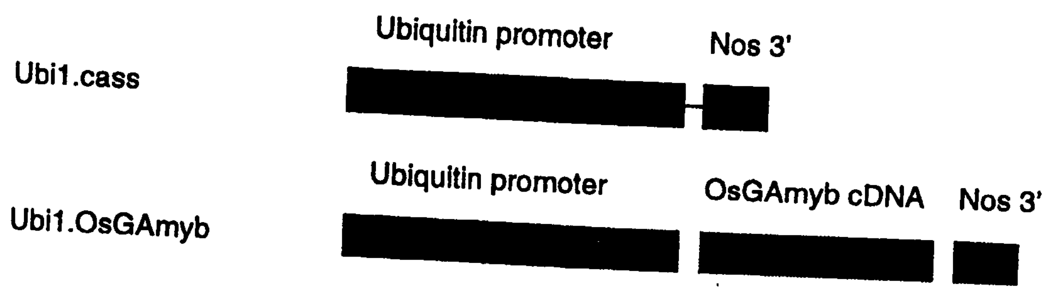

0 1 2 3 6 12
(a) GAMyb
-GA
+GA
0 1 2 3 6 12
(b) α-Amylase
-GA
+GA
0 1 2 3 6 12
(c) RNA
-GA
-GA

Figure 13

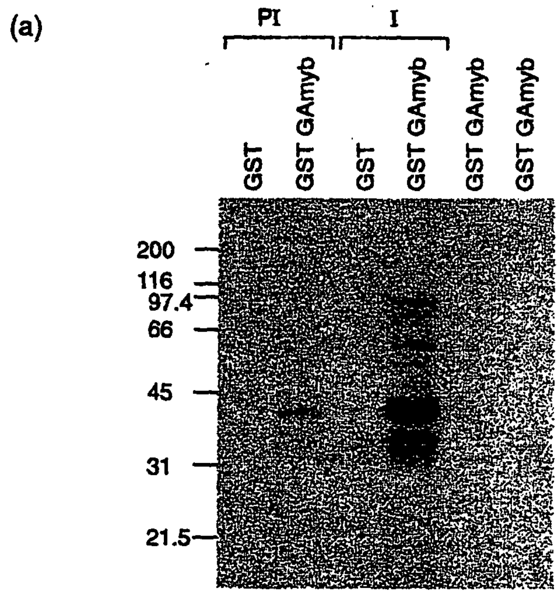
(A) Effector constructs



(B)

	Control	10^{-6} M GA_3
mlo22 + Ubi1.cass	1.7 +- 0.7	100.0 +- 20.5
mlo22 + Ubi1.OsGAmyb	299.6 +- 38.6	320.0 +- 68.6

Figure 14


(a)
PI
I
GST
GST GAmyb
GST
GST GAmyb
GST GAmyb
GST GAmyb
200
116
97.4
66
45
31
21.5

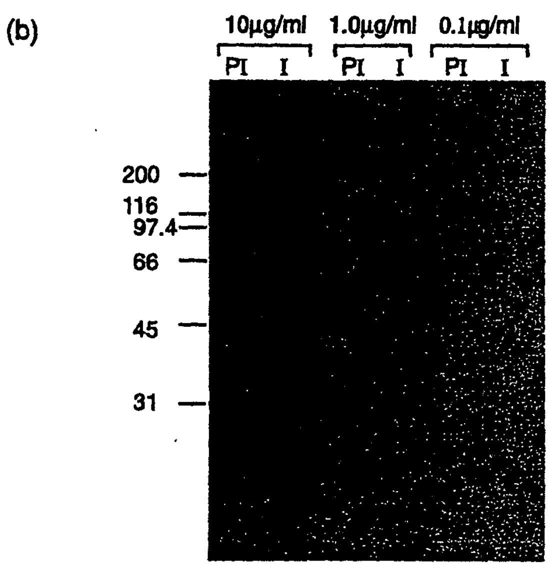

(b)
10μg/ml
1.0μg/ml
0.1μg/ml
PI
I
PI
I
PI
I
200
116
97.4
66
45
31

Figure 15

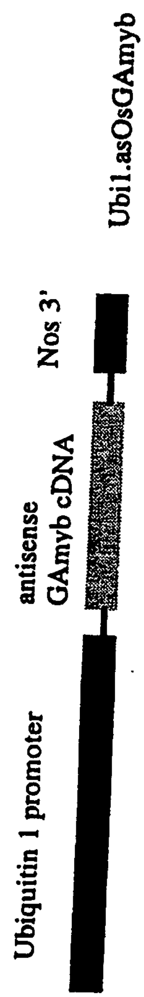

Ubi1.asOsGAmyb
Nos 3'
antisense
GAmyb cDNA
Ubiquitin 1 promoter

Figure 16

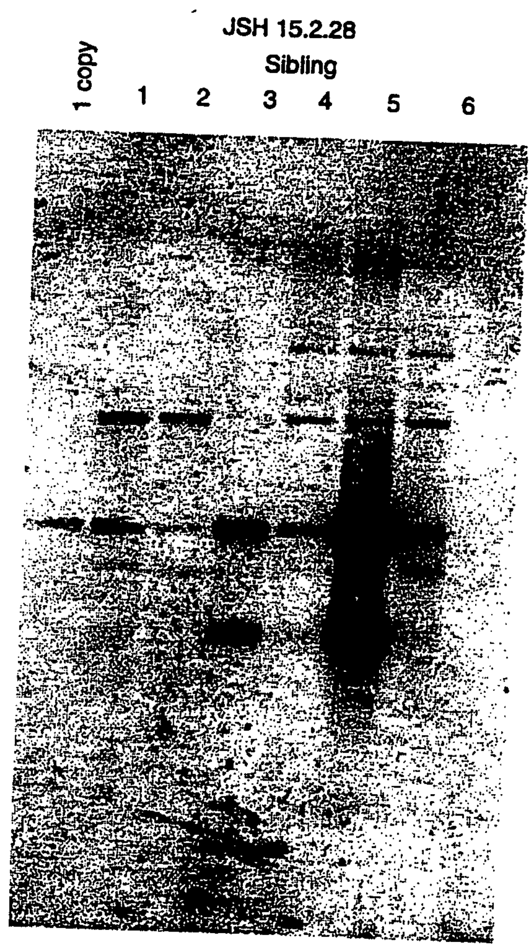


Figure 17

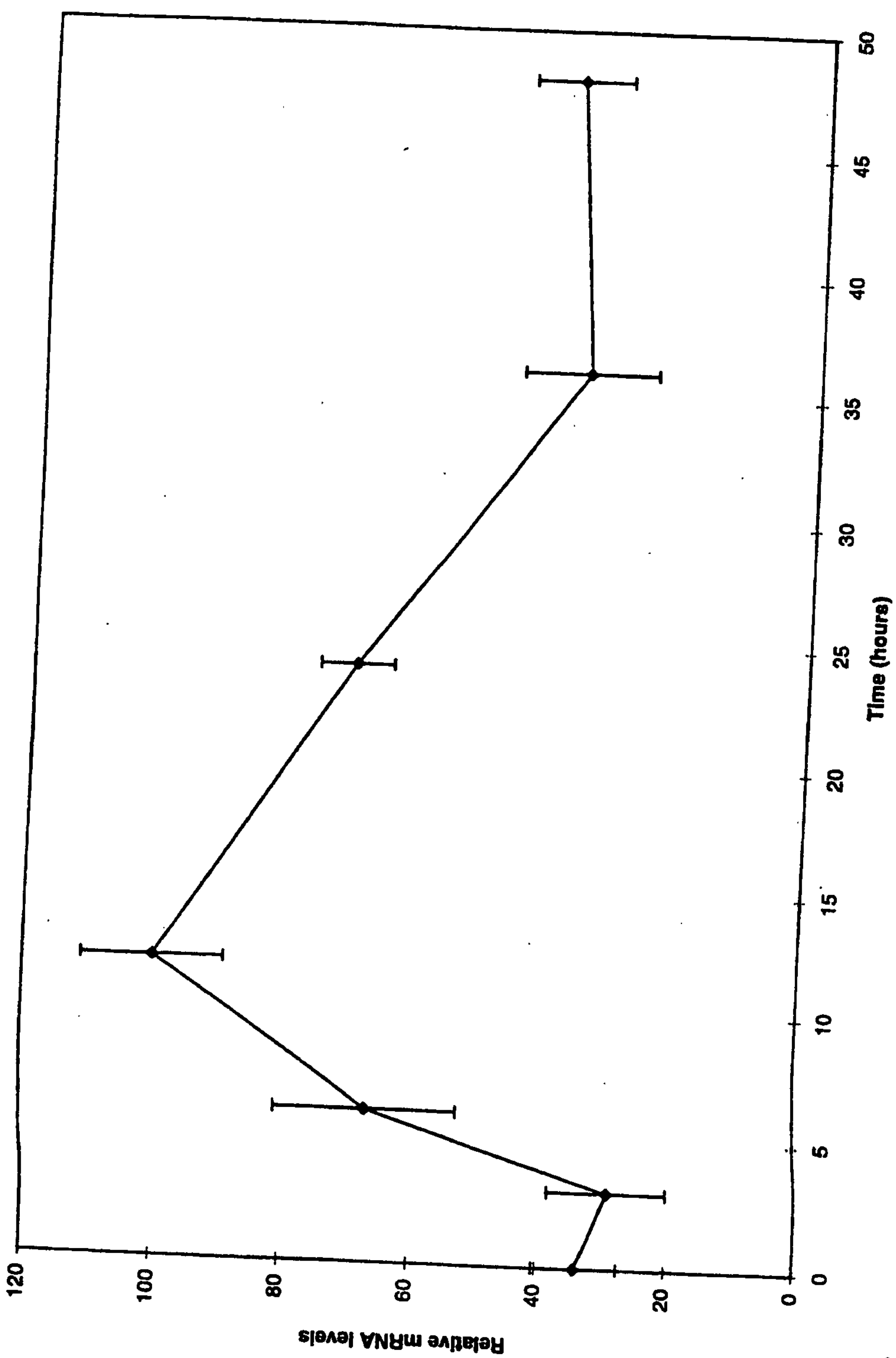

Relative mRNA levels
120
100
80
60
40
20
0
Time (hours)
0
5
10
15
20
25
30
35
40
45
50

Figure 18

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00383

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl^6: C12N 15/29, A01H 1/00, 5/00, C12C 1/00.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
WPAT, CHEMICAL ABSTRACTS, keywords as below.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPM, JAPIO

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DERWENT DATABASES: WPAT, JAPIO+USPM KEYWORDS: MYB: or GAMYB: or HVGAMYB: or OSGAMYB: and (C12N / 1C) or (GENE # or GENE T:) or (GIBERELLI:) CHEMICAL ABSTRACTS: STN SEQUENCE SEARCH and KEYWORDS: MYRVKSESDC/GGAL[NT] GSHAF/ GGAGDTSS/ HPENLRP/ DCEMMHC/SQSFP GIBBERLELLI? MYB? GAMYB? HVGAMYMB? OSGAMYB? ?GAMYB?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	The Plant cell (1995), Vol. 7, No. 11, pg 1879 -1891 (F. Gubler et al), "Giberellin - regulated expression of a myb gene in barley aleurone cells: evidence for myb transactivation of a high - pI.alpha - anylase gene promoter." see whole article.	1-47, 53-55, 63-82.

☐ Further documents are listed in the continuation of Box C

☐ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 August 1996	2 SEP 1996

Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929	CARMELA MONGER Telephone No.: (06) 283 2486